University of Alberta
Printing Department

THE UNIVERSITY OF ALBERTA

A PHARMACEUTICAL STUDY OF BETA-CAROTENE AS A DIAGNOSTIC AGENT

by

VERA M. KOTYLAK

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR

THE DEGREE OF MASTER OF SCIENCE

FACULTY OF PHARMACY

EDMONTON, ALBERTA

OCTOBER, 1966

ABSTRACT

Efforts were made to formulate a stable pharmaceutical beta-carotene preparation, suitable for use as a dosage form in the diagnosis of the malabsorption syndromes.

The rate of oxidation of the oxygen-sensitive carotene was measured under various conditions by a spectrophotometric method to determine the major pro-oxidants to avoid in formulating such a product. Stability of the crystalline material was significantly increased by storage at low temperatures or in a limited oxygen atmosphere. At the concentrations employed, antioxidants were found to be ineffective as stabilizing agents for carotene.

Products formulated included tablets, emulsions, and suspensions. Although film coatings proved to be ineffective in protecting carotene in tablet form, storage of this dosage form in closed containers resulted in good stability for at least a six month period, even at high temperatures. Preliminary clinical testing on a limited number of subjects gave anomalous results with formulations prepared in this laboratory.

Commercially available beta-carotene beadlets, dispersed in orange juice provided an acceptable dosage form which produced reliable results in the number of patients studied.

ACKNOWLEDGMENTS

The author gratefully acknowledges the invaluable assistance and encouragement provided by her supervisor, Dr.A.J. Anderson, during the course of the investigation.

Sincere thanks are also extended to Dr. D.J. Campbell for guidance in clinical studies and suggestions made.

Selection of patients for clinical testing of the products was authorized by Dr. R. Sherbaniuk, Dr. G. King, and Dr. A. Jones. Their assistance and aid in diagnosis of such patients was greatly appreciated.

The author wishes to express sincere appreciation to Mr. W. Jansen for his excellent technical assistance.

Acknowledgement is also made for financial assistance provided by Warner-Lambert, Inc.

The writer also wishes to thank Mrs. Anne Randall for her patience in the typing of this manuscript.

TO MY PARENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The development of reasonably simple and reliable procedures for the confirmation and quantitation of absorptive defects has been responsible for the recent resurgence of interest in the study of the malabsorption syndromes. Approximately two decades ago, it was recognized that low serum carotene levels were characteristic of such conditions (1). Consequently, a measure of the serum carotene has been quite widely accepted as a screening procedure to detect the presence or absence of absorptive defects. A Carotene Tolerance Test, in which beta-carotene functions as a diagnostic agent has been proposed. However, its use has been infrequent.

Beta-carotene is best known for its nutritional value as a vitamin A precursor, and for its use as a coloring agent in the food industry. Due to its interesting chemical structure, and its biological and physiological importance, it has been the subject of numerous investigations. Little attention, however, has been directed to studying the drug as a diagnostic agent. It has been suggested that the unavailability of stable beta-carotene preparations for clinical use has precluded the extensive use of the Carotene Tolerance Test.

SURVEY OF THE LITERATURE

A. Chemistry of the Carotenes

Chemically, beta-carotene is a tetraterpenoid, belonging to the carotenoid class of pigments. It has been found to be distributed widely in both the plant and animal kingdoms. The carotenoids are yellow to red pigments of aliphatic or acyclic structure, composed of eight isoprene units, so linked that the two methyl groups nearest the centre of the molecule are in positions 1:6 while all other lateral groups are in the position 1:5. It is this arrangement which enables cleavage to occur in the centre of the molecule, producing vitamin A. Carotenoids are generally divided into two groups: carotenes which are hydrocarbons, and xanthophylls which are oxygen-containing derivatives (2). All provitamin A carotenoids are structurally related to the parent carotenes, namely β-carotene, α-carotene, γ-carotene, lycopene, δ-carotene, and ε-carotene (3). The first three are the most common, being isomers and having the same empirical composition, $C_{40}H_{56}$. Carotene was first discovered in the roots of carrots by Wackenroder in 1826 (4). Since that time numerous carotenoids have been isolated and identified. Although all possess similar characteristics, they can be separated and identified by their physical and chemical properties.

Beta-carotene possesses two beta-ionone (trimethylcyclohexenyl) rings while other carotenes possess either one or none. The beta-ionone ring is responsible for vitamin A activity, thus beta-carotene possesses a greater degree of biological activity than other carotenes (5).

Willstatter in 1907, established the correct molecular formula of beta-carotene (6). In 1929-1931, Karrer et al elucidated

the structure of this pigment (7). Kuhn and Brockmann in 1932-1935 carried out extensive investigations on beta-carotene and obtained long-chain degradation products, confirming the formula originally assigned (8). Thus, from hydrogenation data, absorption spectra, and degradation products, beta-carotene has been shown to possess the following structural formula:

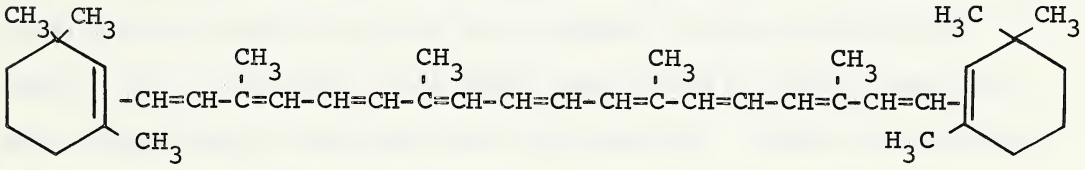


Fig. 1. Structural Formula of β-Carotene

Although the chemical constitution of beta-carotene has long been known, total synthesis of the compound was not achieved until 1950 (9). The color of the synthetic material is seen to vary, depending upon the method of synthesis. Dark violet hexagonal prisms are obtained from benzene-methanol while red-rhombic, almost quadratic plates are obtained from petroleum ether (10). The material is readily soluble in benzene, chloroform, carbon disulfide, and petroleum ether. All carotenoids are fat-soluble. Due to their unsaturated nature, the carotenes are very sensitive to oxidation and autoxidation. By definition, autoxidation refers to an auto-initiated process by atmospheric oxygen, as distinct from the more general term oxidation in which the reaction does not necessarily proceed spontaneously but requires an oxidizing agent (11).

B. Determination and Estimation of Carotenes

In general, physical methods of assaying carotene have employed spectrophotometric or colorimetric measurement of the several carotenoid pigments which have been separated from extracts of samples either by solvent partition or chromatographic adsorption techniques. For the complete identification of a carotene, it has been necessary to carry out a chromatographic purification and to isolate the pigment in the crystalline state (12). Carotenes have been identified by color reactions even though such reactions are non-specific. Dark blue colorations are developed in the presence of concentrated sulfuric acid and the Carr-Price reagent (13). While such color reactions have been found to be useful for qualitative determinations, spectrophotometry is now the standard method used for identification and quantitation of the carotene.

With the advent of spectrophotometric analysis, wide application of the extinction values at wavelengths of maximum absorption has been made for the quantitative determination of carotene* (14). Such procedures have been shown to be accurate and relatively simple. The series of conjugated double bonds of beta-carotene constitutes the chromophoric system. Due to this high coloration, this pigment absorbs readily in the visible region of the spectrum.

Synthetic and naturally-occurring carotene occur in the all trans configuration. This stereochemical form is preserved only when the material is freshly extracted from natural sources, or is kept in the crystalline form. The conjugated double bond

---

* Carotene will refer to beta-carotene unless otherwise indicated.

system of the pigment is subject to cis-trans isomerism, a large number of stereoisomers being possible. The stereoisomers differ from each other in biological potency and in certain physical properties such as adsorption affinity and absorption spectra. A slow but spontaneous cis-trans isomerism of carotene in solution is known to occur (15). Stereochemical alterations are refected by changes in the ultra-violet light absorption curve. Upon oxidation, peaks in the ultra-violet region shift and the general shape of the curve changes with the development of a cis peak, while the spectrum in the visible region retains its original shape (16). Changes in the molecule are indicated by a decrease in peak intensity, i.e., a loss of extinction, and a shift of the spectral bands toward the blue end of the spectrum. Readings in the visible region are used primarily to establish the potency of the material while readings in the ultra-violet region have been used to confirm the absence of stereoisomeric forms of carotene (17).

C. Biochemistry of Carotene

Little detailed information is available concerning the intestinal absorption and metabolism of the provitamin A, beta-carotene. The fat-soluble provitamin is absorbed with relative difficulty due to its low solubility and because it is a hydrocarbon without functional groups in its molecule. Absorption of carotene, which occurs mainly in the small intestine, has been found to be readily affected by several factors.

The nature of the solvents in which the carotene is dissolved is very important in determining the rate and degree of

uptake. Minimal absorption has been found to take place on a fat-free diet while absorption from vegetable oils and fats has been found to be much higher (18,19,20). Recently Roels et al found that a supplement of eighteen grams of olive oil increased absorption five-fold, reflected by large and rapid increases in serum content of carotene (21). An increase in the protein content of the diet resulted in increased carotene absorption, while inclusion of substances that are not digested such as mineral oil and beeswax, have been found to significantly decrease the uptake (22,23).

Thompson showed that if particles were less than one micron in diameter, the provitamin was very effectively utilized, thus indicating the importance of particle size. Apparently microcrystalline beta-carotene embedded in gelatin was remarkably well utilized by the cow, whereas almost no response was obtained for equivalent quantities of carotene dispersed in vegetable oil (24). According to Shaw and Deuel, the rate of absorption is also a function of the concentration of carotene administered and of the surface area of the intestine (25).

Various emulsifying agents have been shown to produce an enhancement of the uptake of the provitamin. When used in small quantities, carotene in an aqueous dispersion containing Tweens* was better absorbed than from oil solutions (26). Lecithin and

---

* Trade Name - Polyoxyethylene derivatives of sorbitan fatty acid esters (Atlas Chemical Industries, Inc., Brantford, Ontario).

bile salts, which possess emulsification properties, have been found to accelerate and intensify absorption (27). The tocopherols, well known for their antioxidant action, are necessary in the protection of carotene from oxidation as it passes through the gastro-intestinal tract. They have been found to be required for normal carotene uptake, and certain levels have been shown to accelerate the absorption (28).

The fate of carotene after absorption is still not completely known, but various theories have been advanced. Moore, in 1929, first demonstrated the conversion of the material into vitamin A and as the vitamin formed accumulated in the liver, it was natural to assume that conversion took place in this metabolically active organ (29). Several workers have since then demonstrated the intestine as the site of conversion (30,31,32). Investigations have indicated that tissues other than the intestine carry out conversion of carotene to the vitamin, but these were found to be much less effective than the liver or intestine (33,34,35).

The mechanism of the transformation is not clearly understood, but two possibilities, as recently reviewed by Olson (36), have been advanced: 1) central fission of the molecule to yield retinene which is then reduced to the vitamin; 2) pathway of attrition by which the vitamin precursor undergoes degradation

of the molecule at one end. Although the central fission hypothesis has been favored, the most recent evidence has strongly suggested that some of the carotene is metabolized by a process other than central fission (37).

After administration, some of the carotene is converted to the vitamin, and both the vitamin and unchanged carotene are transported exclusively by the lymphatic system (38,39). However, the blood stream in general appears to be the pathway of transfer of provitamin A in the animal after absorption has been completed. In the blood it is bound to lipoproteins (40). Cornwell et al, after administering a loading dose of 60 to 200 mg. carotene to normal subjects, found carotene initially in the chylomicrons and S_f10-400 lipoprotein fractions. At a later time after absorption, the pigment was found mainly in the S_f0-10 fraction (41).

While carotene is not stored to any great extent, such storage that does occur, occurs mainly in the liver and fatty depots. It has also been found in various other body tissues (42). The provitamin is not stored to any significant extent in these latter tissues, and apparently the body has no control mechanism to maintain constant blood levels (43).

D. Carotene as a Diagnostic Agent

In 1945, Cayer et al recognized that low serum levels of the fat-soluble vitamins, particularly carotene, were characteristic of the malabsorption syndrome in man (1). By common usage, malabsorption syndromes have been defined as "those diseases associated with impaired digestion and/or absorption of ingested foodstuffs" (44). Primary malabsorption refers to celiac diseases and tropical and non-tropical sprue, while secondary malabsorption refers to all other diseases of the liver, pancreas and intestine that are associated with impaired assimilation. Although a great number of malabsorptive disorders are prevalent, they can be classified into four groups, based upon pathophysiologic mechanisms (45): these include malabsorptive disorders resulting from a) inadequate lipolysis due to lack of lipase or normal stimulation of pancreatic secretions, b) inadequate mixing of food with bile salts and lipase, c) inadequate emulsification due to the lack of bile salts, and d) primary absorptive defects which include biochemical and structural defects of the intestinal mucosal cells. Although steatorrhea is a major manifestation, malabsorption may exist without gross evidence of steatorrhea or diarrhea. Other manifestations include bleeding, tetany, osteomalacia, and/or osteoporosis, malnutrition, edema, amenorrhea, anemia, glossitis and cheilosis, and peripheral neuritis (45).

Many tests have been devised to evaluate the capacity of the gastro-intestinal tract to assimilate foodstuffs. It has been stated that in patients suffering from malabsorption, regardless of the cause, the one part of the diet most likely to be affected is fats. Consequently a measure of fat absorption

has been used to diagnose the presence of malabsorption (46). As indicated previously, carotene, being fat-soluble is apparently absorbed by the same route as fats, therefore malabsorption of fats is reflected by the malabsorption of carotene. Parker and Ross in 1960 surveyed 230 essentially well patients. Fasting serum carotene levels were determined in each as a screening procedure. They found five asymptomatic patients in whom gross steatorrhea was present, indicating the value of serum carotene in the detection of malabsorption (47).

In studies by Wenger et al, a rapid determination of fasting serum carotene levels was found to be effective as a screening test in 103 of 110 patients (48).

Normal serum levels range from 70 to 282 mcgm./100 ml., but may vary with age, sex, season, and geographical location. These levels have generally been employed in a screening test, being valuable in the early detection of malabsorption syndromes. Very high levels are indicative of carotenemia while low levels may reflect dietary depletion or impaired absorption (46). To differentiate between the two latter possibilities, a Carotene Tolerance Test, as initially proposed by Adlersberg in 1949 (27), is performed. A loading dose of carotene administered orally will increase serum carotene levels in normal patients but will have little effect on levels in patients afflicted with a malabsorptive condition. The Carotene Tolerance Test is useful clinically as the diagnostic agent is naturally present in the blood, and is non-toxic at high levels (49).

The Carotene Tolerance Test was first proposed in 1949 as an alternative to the Vitamin A Tolerance method, then in

general use. At that time, Adlersberg et al studied factors involved in the absorption of carotene in man (27). They found 120 mg. carotene in 24 gm. of butter to be a suitable dose for the attainment of an optimal although slow elevation of carotene concentration in the serum. These workers suggested a disadvantage in the test in that it required a period of at least three days and often as long as seven days. This has since been disputed however, as studies at the University of Alberta Hospital in Edmonton have yielded data which indicate that the test may be completed satisfactorily in twenty-four hours (50).

In studies by Wenger et al, seventeen patients with low serum carotenes were given 120 mg. carotene in oil to determine if the low levels were due to malabsorption or dietary depletion. Correct diagnoses were made in each case studied (48).

Feldman and Adlersberg studied the response to carotene loading in malabsorption and found it to be as good or better than the Vitamin A Tolerance method previously used. This loading procedure was effective in diagnosing the condition in all patients investigated. Sixteen patients with malabsorption syndromes did not absorb the provitamin while nine healthy control subjects gave a significant rise (51).

As a diagnostic agent, carotene has usually been administered with a large quantity of butter or margarine. An aqueous dispersion of carotene administered with forty to eighty grams of a lipid test meal or protein-carbohydrate test meal have also been used (40).

Besides the serum carotene test, other tests currently available for screening and diagnosing malabsorption include:

1) Direct examination of the stool in which gross and microscopic examinations of the stool are performed to detect oil droplets and buttery material present, 2) Fat Balance study. Here, 100 gm. fat are administered to a subject. If chemical analysis of the stool indicates more than 6 gm. of fat per 24 hours is excreted, malabsorption is indicated, 3) I^{131}-labeled triolein and 4) I^{131}-labeled oleic acid. Methods 3 and 4 involve oral administration of isotopically labeled fat with subsequent determination of radioactivity in the blood and stool, 5) Chylomicron count. This is essentially a dark field examination of serum to determine whether the chylomicrons are present in a normal range. 6) Serum Turbidity Test, where an increase in the optical density of 0.1 to 0.2 of the serum is considered a normal elevation, and 7) Vitamin A Tolerance Test. After ingestion of the vitamin in an oil vehicle, a marked increase in the vitamin A content of the blood occurs in normal subjects (45,52).

The majority of these tests suffer disadvantages in that they are either tedious, time-consuming, expensive or unreliable. The Carotene Tolerance Test is preferred by the University of Alberta Hospital, Edmonton, as it has been found to be simple, inexpensive and sufficiently reliable.

E. Oxidation of Carotene

Theoretically, all organic compounds can oxidize, with some substances being more sensitive in this respect than others. Carotene, due to the presence of the large number of unsaturated double bonds, is very subject to autoxidation. Consequently, when used as a diagnostic agent, precautions must be taken to ensure that the carotene is administered in its pure form.

As indicated earlier, autoxidation is an auto-initiated oxidation by molecular oxygen (11).* The mechanism involved and the primary products of autoxidation of carotene are not completely known, but kinetic and chemical studies carried out with other unsaturated compounds containing conjugated double bonds have led to the conclusion that cyclic or polymeric peroxides of the dialkyl type constitute the primary degradation products of these compounds and that furthermore, oxidation proceeds by the propagation of radical chains (53). By analogy, carotene has been assumed to follow a similar mechanism. Carotene, however, is much more rapidly attacked by oxygen than the fatty acids due to the greater number of double bonds. "The general belief that oxidation takes place through propagation of free-radical chains would appear to be based on the inhibitory action of antioxidants and the detection of peroxides in the autoxidizing carotene" (53).

Oxidation in general terms, is thought to proceed by the following mechanism (54):

(1) Initiator $\longrightarrow$ $R^{\cdot}$ (free radical)

---

* Oxidation will refer to autoxidation for convenience.

The free radical reacts rapidly with oxygen to give an alkyl-peroxy radical. The peroxy radical may either abstract an allylic hydrogen atom from another molecule or add to the double bond. Because carotene contains double bonds, the following chain of reactions, known as the Propagation Step probably occurs:

$$\longrightarrow \quad R^{\cdot} + O_2 \longrightarrow ROO^{\cdot} \text{ (peroxy radical)} \qquad (2)$$

$$ROO^{\cdot} + C{=}C \longrightarrow ROOC\text{-}C^{\cdot} \qquad (3)$$

and/or

$$ROO^{\cdot} + R\text{-}H \longrightarrow ROOH + R^{\cdot} \qquad (3a)$$

Kinetic chains are broken by the removal of the chain-propagating radicals, leading to the termination of the reaction:

$$R^{\cdot} + R^{\cdot} \longrightarrow \qquad (4)$$

and/or

$$ROO^{\cdot} + R^{\cdot} \longrightarrow \quad \text{Non-radical (inactive) products} \qquad (5)$$

and/or

$$ROO^{\cdot} + ROO^{\cdot} \longrightarrow \qquad (6)$$

Autoxidation may be initiated by light, heat, peroxides, or heavy metals. Although the mechanism for carotene has not been proven, some of the secondary oxidation products have been identified (55).

F. Stabilization of Carotene

It has been shown that oxidation proceeding by chain reactions may be changed radically by the addition of extremely small amounts of substances of either positive or negative catalytic effect (56). The 'negative catalysts' are antioxidants which break up the chain during oxidation. They are not proper catalysts as they themselves are used up during the process. Usually the substances which act as, and are used as antioxidants are easily oxidizable compounds. They act on the principle of a single molecule of antioxidant preventing the progress of the reaction by reacting with one or more of the compounds involved in the chain reaction and thus impeding a long chain of reactions (57). To keep chain reactions going, a reactive radical R° or ROO° is needed (Equations 2,3a). An antioxidant AH has the ability to react with these radicals, thereby forming the radical A° which is not sufficiently reactive to sustain the chain reaction. Two possible chain-ending reactions involving the antioxidant are:

$$ROO^{\circ} + AH \longrightarrow ROOH + A^{\circ}$$

or

$$R^{\circ} + AH \longrightarrow RH + A^{\circ}$$

The terminal reaction is thought to proceed as follows:

$$\left.\begin{array}{ll} A^{\circ} + A^{\circ} \longrightarrow & \\ \text{and/or} & \\ RH + A^{\circ} \longrightarrow & \\ \text{and/or} & \\ R^{\circ} + A^{\circ} \longrightarrow & \end{array}\right\} \text{non-radical products}$$

Not until all of the antioxidant has been used up does the oxidation proper commence and then frequently at a rapid rate. Antioxidants retard or delay oxidation; they do not provide total prevention. Addition of an antioxidant will have little or no effect when a substance has been partially oxidized, because when the chain reaction is in full swing and many free radicals have been formed, all antioxidant molecules will be rapidly destroyed (54). Antioxidants function either by breaking the chain reaction, or by preventing the introduction of chain-initiating radicals into the system. Phenol and amine antioxidants have experienced the widest use.

Among the commonly used phenolic antioxidants are the gallates, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), nordihydroguaretic acid (NDGA), and the tocopherols. These phenols are permitted in foods in Canada and in the United States (58) at certain maximum concentrations. While N,N-diphenyl-p-phenylenediamine (DPPD) is also a commonly used amine antioxidant, its use in food is not allowed in Canada (59).

High et al, in studies related to the effect of antioxidants on the metabolism of carotene and vitamin A, showed that the antioxidants tested did not interfere with absorption of carotene as such, but might possibly interfere with the conversion to the vitamin (60). For this reason, it was felt that antioxidants used to stabilize carotene should not affect the value of the diagnostic agent.

In addition to the use of antioxidants, a number of other factors were known to have a beneficial effect on the prevention of oxidation: 1) exclusion of light, 2) avoidance of high

temperature, 3) freedom from peroxides and heavy metals, and 4) minimal exposure of surface area to the atmosphere (54).

Stability studies on carotene have been performed mainly on forage, food and dairy products, and dilute carotene solutions. Since carotene occurs naturally in forage, studies have dealt mainly with the problem of stabilizing the naturally-occurring pigment. Such reports have indicated that the rate of oxidation can be slowed at low temperature (61,62,63). Displacement of atmospheric oxygen with an inert gas such as nitrogen or carbon dioxide was found to decrease carotene loss significantly (64,65). Some protection from contact with atmospheric oxygen was also afforded by pelleting and coating the material with wax (66). Wall and Kelly investigated the effects of temperature, concentration of carotene and source of carotene in relation to the stability of the compound (63). They showed that stability increased with increasing concentration and decreasing temperature, and that the stability varied with the carotene source. Apparently carotene stability is associated with source, as it was shown that synthetic beta-carotene showed more deterioration during storage in petroleum ether than natural alpha or beta-carotene (67).

The use of antioxidants in delaying oxidation of both synthetic and natural carotene has produced varying results. The majority of these studies have been carried out on complex systems such as forage, food and oils. Thompson, in studying fifty-four compounds as to their antioxidant activity in dehydrated alfalfa meal, found hydroquinones and diphenylamines to offer the best protection of carotene (68). Carotene has been stabilized in

oil solutions by various phenolic and amine antioxidants. NDGA has given useful protection in various vegetable oils, fish oils and dairy products, and DPPD has been found to be effective in animal feeds (69-72). In solvents free of antioxidants, the addition of a stabilizing agent was found to have a strong protective effect. In solvents containing natural antioxidants, little additional effect was observed (69,73). Among the common vegetable oils, cottonseed oil was found to be outstanding as a stabilizing solvent for carotene (74).

In mineral oil solutions, a number of gallates were found to extend the induction period for carotene almost a hundred-fold, but when used in alfalfa meal they appeared to have a negligible effect (75). Bickoff found DPPD to be effective in coconut oil, lard, and mineral oil, but devoid of activity in cocoa butter and cottonseed oil (76,77). Tocopherol was seen to be effective as an antioxidant for carotene in mineral oil, but exerted no appreciable effect in cottonseed oil (78). Thus, it became obvious that antioxidant effectiveness could not be predicted in different systems. Consequently, each antioxidant must be tested in each system in which it is to be used.

Various investigators have demonstrated the effect of fats on the protection of carotene. These experiments illustrated the dual effect of unsaturated fats in promoting carotene oxidation, while at the same time making antioxidants more effective, presumably by improving their contact or orientation with respect to carotene (79-81). It has been shown that the addition of fats to carotene has a large pro-oxidant effect, this effect increasing with increasing degree of unsaturation (53).

In general, stability of carotene was seen to vary with concentration of carotene (82), purity of the carotene (83), concentration of antioxidant (naturally present or added), and surface-volume relationship of carotene-oil preparations (71). Since the solubility of carotene in vegetable oils is only 0.05-0.01%, at ambient temperature (84), most of the studies have been performed on very dilute solutions. Dilute solutions of carotene have been found to be more stable than concentrated ones (53).

The commercial synthesis of carotene and the stable synthetic carotenoids has opened a new field of research concerned with the use of these pigments. To date, these compounds have been considered only as food colorants (85). As concentrated carotene products, however, they may be applicable for use in the Carotene Tolerance Test. A variety of these products are available, consisting of beta-carotene liquid suspensions, semi-solid suspensions, and gels. They are stable only in evacuated containers or in an inert atmosphere. Thus it appears that stabilization by antioxidants has not been accomplished when carotene is present in large amounts. A study of the literature revealed one product, in the form of water-dispersible beadlets, which was claimed to offer good stability (86). Apparently the beadlets have been protected from attack by atmospheric oxygen by means of a sugar-gelatin matrix.

STATEMENT OF THE PROBLEM

Although determination of serum carotene has been used widely for screening purposes in the detection of the malabsorption syndromes, the value of the Carotene Tolerance Test as an aid in diagnosis has not been confirmed (45). This test is claimed to offer many advantages: it is reasonably simple to perform and relatively inexpensive; it enables the physician to establish normal controls in his own laboratory rather than to rely on the work of others. The University of Alberta Hospital has adopted this technique to diagnose the presence or absence of absorptive defects in patients who display low serum carotene levels. The test has been found to be extremely useful in the majority of cases studied in the past three years. One of the disadvantages associated with the test, however, is the inherent instability of the diagnostic agent, beta-carotene. It is believed that if this stability problem were solved, the test would experience increased popularity in other hospitals.

On studying the handling procedure for the drug as employed at the University of Alberta Hospital, it was found that the material was first transferred to clear gelatin capsules, the contents of each capsule constituting a loading dose. Storage conditions were not strictly defined and the possibility of prolonged ward storage at room temperature could not be ignored.

At the time of administration, the contents of a capsule were mixed with approximately twenty grams of butter (two "pats"). This mixture was spread on bread and consumed by the patient. The disadvantages of the method were immediately apparent. The major disadvantage was the variability in potency of the carotene

due to the lack of detailed control of storage conditions, followed by the lesser problem of inefficiency and inaccuracy of the method of administration. Consequently, it was considered of interest to investigate methods of increasing the stability of the organic compound, to improve the method of administration, and also to develop more acceptable dosage formulations with subsequent clinical evaluation of each.

Thus the investigations carried out in this laboratory and the University of Alberta Hospital were divided into three phases:

a) Evaluation of factors affecting stability of carotene in the crystalline state

b) Stability studies on selected carotene formulations

c) Clinical evaluation of selected dosage forms.

EXPERIMENTAL

A spectrophotometric procedure was used in all studies as a method of determining the quantity of pure beta-carotene present. Quantities were expressed either as extinction coefficients or as weight of the unchanged material.

A typical absorption curve for carotene in chloroform is presented in Figure 2. Chloroform was the solvent primarily used, the absorption maxima of carotene occurring at 497 and 466 mμ. Concentrated solutions of carotene in chloroform produced a deep orange-brown color, while dilute solutions were yellow colored. Such solutions were crystal clear. Quantitative measurements of carotene were determined spectrophotometrically by direct dilution of carotene in the solvent. Where necessary, carotene was extracted from the systems by suitable techniques. The decrease in peak height at 466 mμ. was used as a measure of the rate of oxidation (53).

Preliminary work involved separating oxidation products from the pure drug by column chromatography in various samples which had undergone different degrees of oxidation. Alumina columns with petroleum ether as the eluting agent were used initially (82). Magnesia columns with n-hexane as the eluting solvent were later employed to verify these results (87). Under the experimental conditions employed, colored oxidation products constituted only a small fraction of the carotene remaining. Therefore, since no significant difference in color intensity occurred after chromatography, it was considered unnecessary to remove degradation products from each sample before spectrophotometric analysis. Thus absorptiometric measurements could be carried out directly on oxidized samples simply by diluting the sample in the

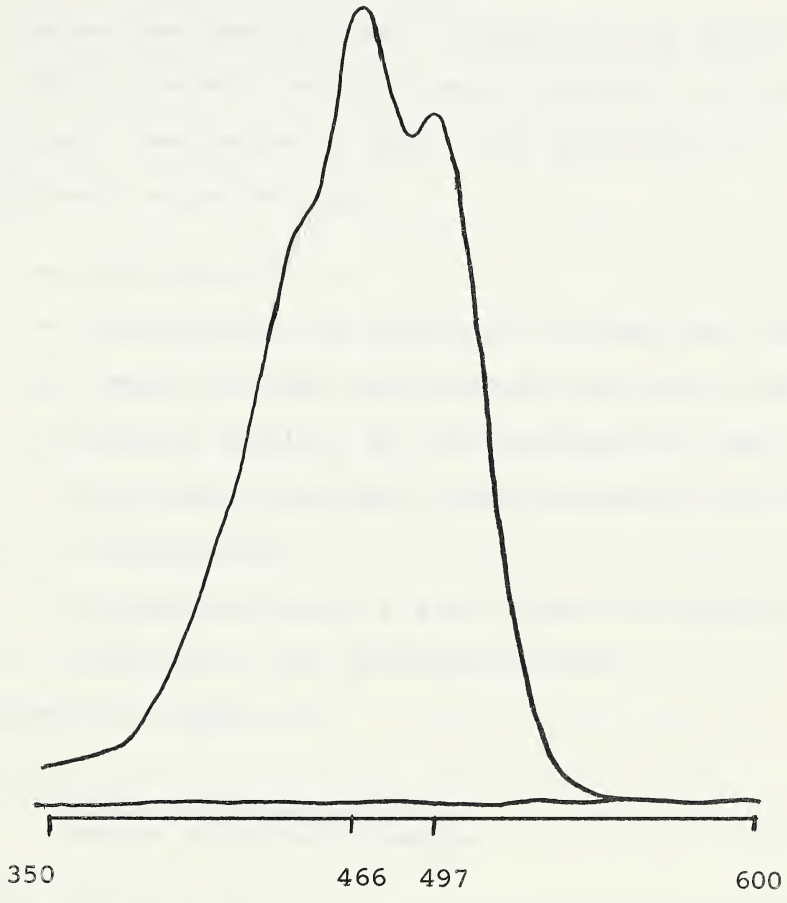


Fig. 2. Absorption Spectrum of All-*trans* β-Carotene

solvent. Budowski and Bondi followed a similar procedure in studying autoxidation of carotene (53).

In the following investigations, carotene samples were dissolved in chloroform and then diluted. Absorbance was determined directly. Optical density readings were converted to the extinction coefficient, designated as $E^{1\%}_{1cm.}$ The extinction is defined by the following equation (14):

$$E^{1\%}_{1cm.} = 1/cd \log 1/T$$

where c = concentration of substance in grams per 100 mls.

d = length of light path through solution in cm.

$\log 1/T$ = optical density, or the reciprocal of the fraction of incident light transmitted through the solution.

$E^{1\%}_{1cm.}$ = the extinction of a 1 cm. layer of a 1% w/v solution of the absorbing solute.

$E^{1\%}_{1cm.}$ was calculated here simply as:

$$E^{1\%}_{1cm.} = \frac{\text{O.D. x dilution factor}}{\text{weight in grams of sample}}$$

Throughout the test procedure, carotene weights were obtained from a standard curve. The standard solution was prepared by dissolving 50 mg. of carotene in 50 ml. of chloroform. A calibration curve was prepared from this standard by appropriate dilution. The standard solutions were prepared at the time of calibration, as such solutions were not stable under ordinary conditions for any significant period of

time. Calibration curves were prepared whenever the $E^{1\%}_{1cm.}$ of different samples varied. Such curves were found to obey Beer's Law, both when high and low O.D. values were employed. Figure 3 represents a typical carotene standard curve.

Initially, beta-carotene stocks were obtained from three different sources. Subsequent assay of these samples indicated that the $E^{1\%}_{1cm.}$ was not the same for each. Extinction values of 2125 to 2450 were obtained from the different sources. It was apparent that certain lots had suffered some degree of oxidation. One vial contained material which displayed a value of 1418. As a result, it was decided to restrict further purchases to one supplier*. The carotene was supplied in nitrogen-sealed five gram vials. For each study, a freshly opened vial was used.

The solubility of carotene in various solvents was determined in order to select the preferred assay system. On the basis of handling characteristics and maximum solubility, chloroform was found to be the solvent of choice. As a precautionary measure, an ACS grade of chloroform was employed. This was, in turn, purified by redistillation after washing with 1N potassium hydroxide and drying over calcium chloride.

A recording double beam spectrophotometer was used for all determinations.+

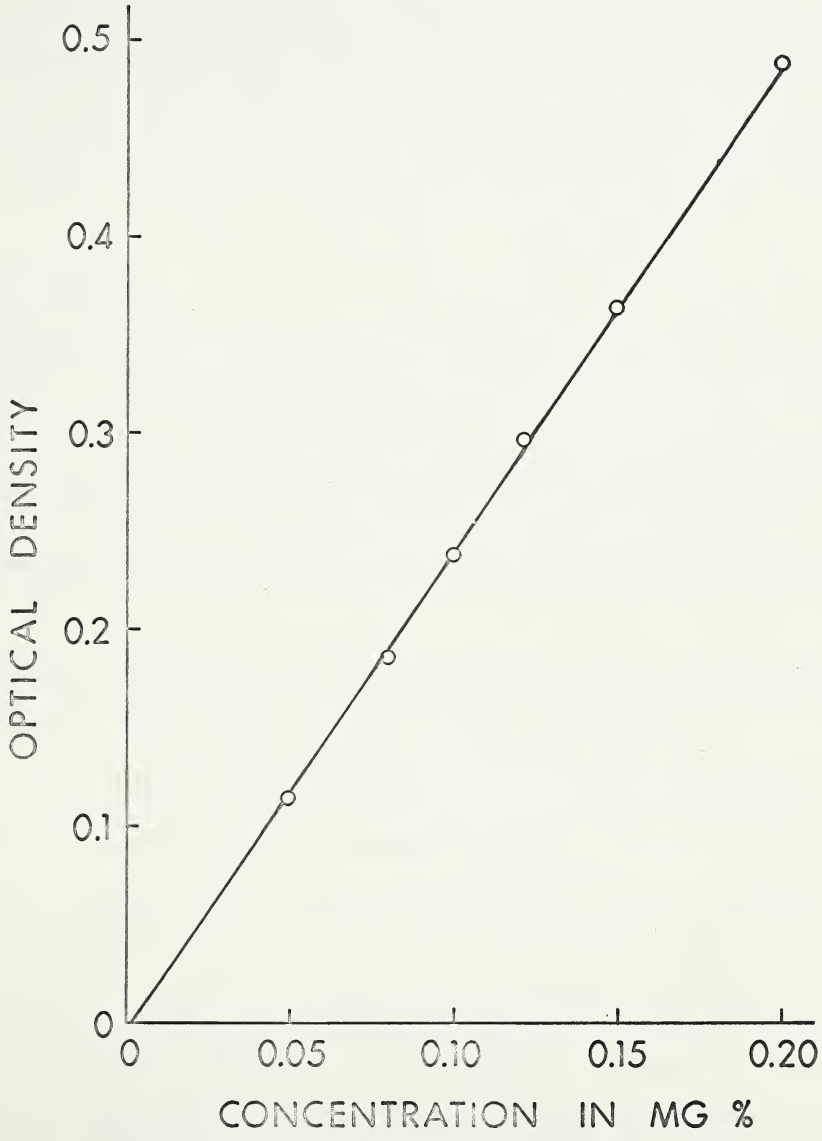
---

* Distillation Products Ind., Division of Eastman Kodak Co., Rochester 3, New York.

\+ Beckman Model DK-2.

Fig. 3

Relationship between Optical Density at 466 mμ. and Concentration of Beta-Carotene in Chloroform


0.5
0.4
0.3
0.2
0.1
0
OPTICAL DENSITY
0
0.05
0.10
0.15
0.20
CONCENTRATION IN MG %

A. Solid State Stability of Crystalline Carotene

To increase the shelf-life of an oxidizable compound, it is necessary to determine the major factors initiating the oxidation process so that they may be avoided, if possible. In an effort to determine the degree of instability of solid beta-carotene as affected by environmental conditions when stored as a diagnostic agent, common pro-oxidants were evaluated as to their effects.

1. Temperature

In the University of Alberta Hospital, it was reported that capsules of carotene stored in the refrigerator retained their potency for longer periods than those kept at room temperature. To obtain data relating the sensitivity of the pigment to temperature changes, carotene stability was studied at different temperatures.

Samples of 10 mg. of crystalline carotene were weighed into 1 dram vials. The vials, open to air, were placed in an oven at 50°C., in an oven at 37.5°C and in a refrigerator at 5°C. Vials were also stored at ambient temperature in a dark cupboard. At intervals, sample vials were withdrawn and their contents transferred quantitatively into a 100 ml. volumetric flask. All samples were diluted so that the final concentration was 1 mg. solute in 500 mls. solvent. Results, illustrated in Figure 4, Table I, indicated a very significant effect of temperature on carotene stability. Oxidation was seen to commence at very nearly the same rate with all samples, but progressed much more rapidly at higher temperatures. Stability varied inversely with temperature,

TABLE I

[a] Samples (10 mg.) were stored in open vials.

[b] Optical density of carotene in chloroform was determined at 466 mμ. Initial O.D. was .490.

[c] Wt. indicates the weight of the active material present and is expressed in mg. per vial.

[d] Loss indicates the average loss in per cent of the active material.

TABLE I

Effect of Temperature on Stability of Crystalline Beta-Carotene[a,e]

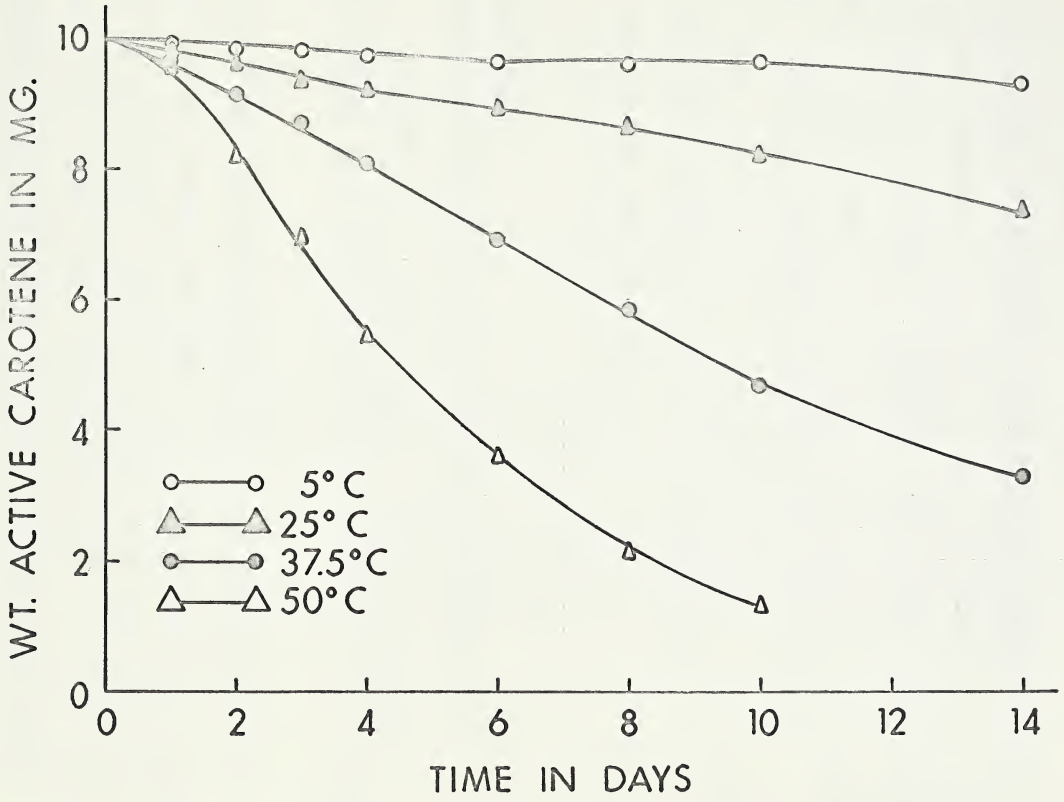
Time in Days	5°C			25°C			37.5°C			50°C		
	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]
1	.480 .480	9.8 9.8	2.0	.475 .475	9.7 9.7	3.0	.470 .475	9.6 9.7	3.5	.469 .470	9.6 9.6	4.0
2	.480 .480	9.8 9.8	2.0	.470 .470	9.6 9.6	4.0	.448 .450	9.1 9.2	8.5	.400 .400	8.2 8.2	18.0
3	.480 .480	9.8 9.8	2.0	.460 .458	9.4 9.3	6.5	.420 .430	8.6 8.8	13.0	.340 .340	6.9 6.9	31.0
4	.473 .474	9.7 9.7	3.0	.450 .450	9.2 9.2	8.0	.394 .395	8.0 8.1	19.5	.270 .270	5.5 5.5	45.0
6	.475 .470	9.7 9.6	3.5	.440 .437	9.0 8.9	10.5	.340 .340	6.9 6.9	31.0	.190 .193	3.9 3.9	61.0
8	.470 .470	9.6 9.6	4.0	.428 .430	8.7 8.8	12.5	.293 .289	6.0 5.9	40.5	.105 .105	2.1 2.1	79.0
10	.470 .470	9.6 9.6	4.0	.410 .408	8.4 8.3	16.5	.232 .229	4.7 4.7	53.0	.070 .080	1.4 1.6	85.0
14	.453 .458	9.2 9.3	7.5	.360 .363	7.3 7.4	26.5	.160 .165	3.3 3.4	66.5	-	-	-

[e] For added convenience, O.D. values were expressed in terms of $E^{1\%}_{1cm.}$ (see Appendix Table I)

Fig. 4

Effect of Temperature on Stability of

Crystalline Beta-Carotene


WT. ACTIVE CAROTENE IN MG.
10
8
6
4
2
0
0
2
4
6
8
10
12
14
TIME IN DAYS
5° C
25° C
37.5°C
50° C

as indicated by decreasing reaction rates as temperature was decreased. These results were in agreement with published work indicating temperature as the major factor to consider in storing various forms of carotene (61).

Prior to the above experiment, preliminary studies were performed on 200 mg. samples of carotene stored at the indicated temperatures in screw-cap vials. Aliquots were withdrawn periodically. However, satisfactory replicates could not be obtained by this method, since it appeared that the same carotene in different containers was oxidized at different rates. This was attributed to the free oxygen present in the containers, as regulated by the closure on the vial.

2. Atmospheric Oxygen

Since preliminary studies indicated varying rates of decomposition of carotene in closed vials, it was considered advisable to determine the sensitivity of the material to atmospheric oxygen.

Crystalline carotene was weighed into two dram screw-cap vials and stability was observed under the following conditions: a) samples were maintained in direct contact with the atmosphere; b) samples were stored in closed, screw-cap vials. As an added precaution, aluminum foil was used as a preliminary seal with final closure being effected by regular screw caps.

All samples were stored at room temperature and assayed weekly to determine reduction in potency. Results of this study, shown in Figure 5 and Table II, indicated the importance of maintaining a limited oxygen atmosphere. Apparently, the

TABLE II

Effect of Atmospheric Oxygen on Stability of Crystalline Beta-Carotene[a,e]

Time in Weeks	Carotene Exposed to the Atmosphere			Carotene Stored in Closed Vials		
	O.D.[b]	Wt.[c]	loss[d]	O.D.[b]	Wt.[c]	loss[d]
1	.470	95.9		.472	96.3	
	.463	94.5	5.0	.472	96.3	3.7
	.463	94.5		.472	96.3	
2	.440	90.0		.445	90.8	
	.430	87.7	11.6	.440	90.0	9.7
	.430	87.7		.440	90.0	
3	.400	82.0		.410	83.7	
	.400	82.0	18.6	.410	83.7	16.3
	.394	80.4		.410	83.7	
4	.340	69.4		.400	82.0	
	.338	69.0	30.2	.400	82.0	18.0
	.348	71.0		.400	82.0	
5	.234	47.8		.395	80.6	
	.236	48.2	51.3	.392	80.0	19.7
	.245	50.0		.393	80.2	
6	.182	37.2		.381	77.7	
	.191	38.9	61.6	.382	77.9	22.1
	.192	39.1		.383	78.1	

[a] Samples (100 mg. each) were stored at room temperature in two dram glass vials.

[b] Optical density of carotene in chloroform was determined at 466 mμ. Initial O.D. was .490.

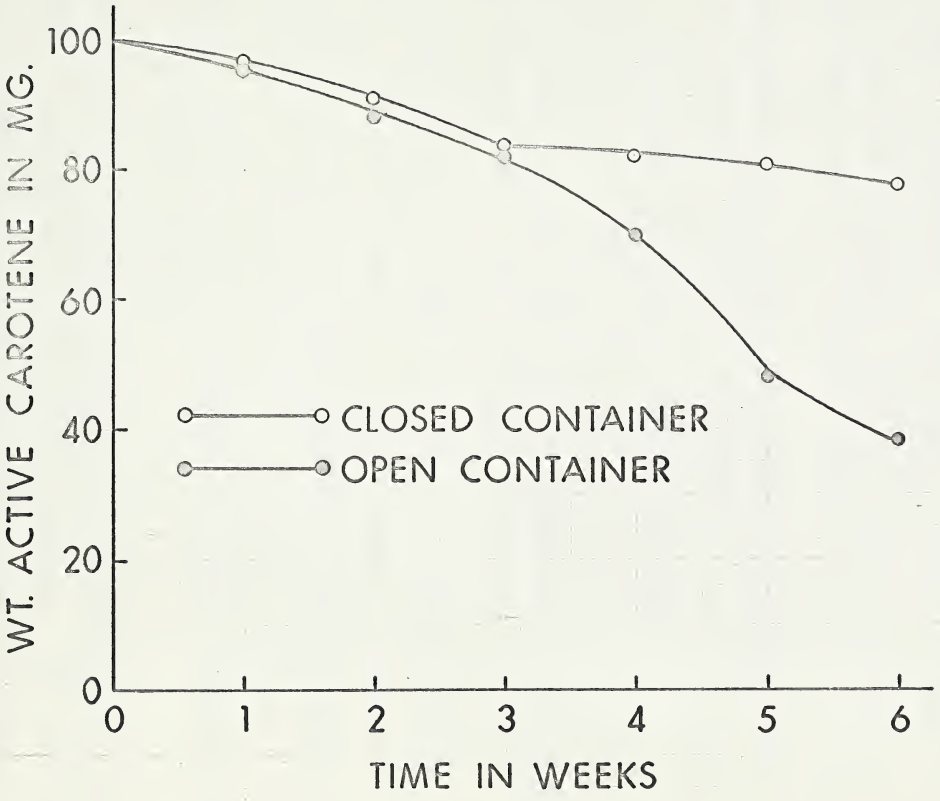
[c] Wt. indicates the weight of the active material present and is expressed in mg. per vial.

[d] Loss indicates the average loss in per cent of the active material.

[e] For added convenience, O.D. values were expressed in terms of $E^{1\%}_{1cm}$. (See Appendix Table II).

Fig. 5

Effect of Atmospheric Oxygen on Stability of Crystalline Beta-Carotene


WT. ACTIVE CAROTENE IN MG.
100
80
60
40
20
0
0
1
2
3
4
5
6
TIME IN WEEKS
CLOSED CONTAINER
OPEN CONTAINER

closure was effective to some extent in protecting carotene from the atmospheric oxygen.

3. Diluent

Beta-carotene is known to be a natural constituent of forage. When mixed with various carriers, it was found to undergo oxidation at different rates (63), presumably due to the effect of stabilizing agents present, or to a protective effect by which the effective surface of the carotene exposed to the air was minimized. It has also been found that some diluents, once regarded as inert ingredients, may potentiate the chemical degradation of active ingredients (88).

The University of Alberta Hospital employed carotene as a diagnostic agent in the form of a carotene-lactose powder mixture. It was felt that the reported instability of this dosage form could be due, in part at least, to the presence of the lactose. For this reason it was decided to determine the effect of lactose and four other commonly used diluents on crystalline carotene. Materials chosen included lactose, kaolin, calcium carbonate, and calcium phosphate, tribasic. Crystalline carotene was used as the control.

Each powder was intimately mixed with carotene in a 2 to 1 ratio to magnify any changes that might develop. The mixtures were packaged in clear gelatin capsules and stored at room temperature. An accurately weighed quantity of each sample was later added to chloroform. The undissolved diluent was removed by filtration and the clear filtrate was made up to volume with more chloroform. The adjusted filtrate was then assayed spectro-

photometrically. From the results obtained (Table III), no significant influence on the rate of oxidation of carotene could be attributed to the presence of the diluents studied. Each system exhibited a normal oxidation curve complete with the initial induction period. Therefore, it was concluded that instability was due entirely to the oxidative process without initiation and/or acceleration by diluents.

4. Light

Another factor considered as a possible potentiator of oxidation in the system presently employed was photodecomposition. Since light is a common pro-oxidant in oxidation processes, the possibility of using colored gelatin capsules to prolong the induction period was investigated. Red, brown and clear capsules* were used, where the clear gelatin capsules should allow light transmittance while the effect of light would be minimized with the more selective screening by the colored capsule. Since it is known that photochemical activity of light radiation drops off with increasing wavelength, it could be expected that the colored capsules would afford better protection of the product against light than the clear capsules. Figure 6 represents the absorption spectra of the dyes used in the red and brown capsules. A 0.15% w/v gelatin solution, prepared by dissolving clear gelatin capsules in water, served as a blank.

---

* Courtesy of Eli Lilly Company, Ltd., Indianapolis, Indiana.

TABLE III

Effect of Diluents on Stability of Crystalline Beta-Carotene[a,e]

Time in Weeks	Control			α-Lactose			Kaolin			Calcium Carbonate			Calcium Phosphate		
	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]
1	.810 .810	42.9 42.9	4.7	.814 .812	43.1 43.0	4.3	.808 .810	42.8 42.9	4.8	.820 .820	43.4 43.4	3.6	.823 .820	43.6 43.4	3.3
2	.608 .610	32.2 32.3	28.3	.610 .607	32.3 32.1	28.5	.630 .620	33.3 32.8	26.5	.604 .600	31.9 31.7	29.3	.628 .628	33.2 33.2	26.2
3	.510 .500	27.0 26.5	40.6	.495 .495	26.2 26.2	41.8	.498 .488	26.4 25.8	42.0	.532 .523	28.2 27.7	37.8	.472 .471	25.0 24.9	44.5
4	.330 .321	17.5 17.0	61.7	.346 .347	18.3 18.4	59.2	.331 .327	17.5 17.3	61.3	.364 .366	19.3 19.4	57.0	.330 .333	17.5 17.6	61.1

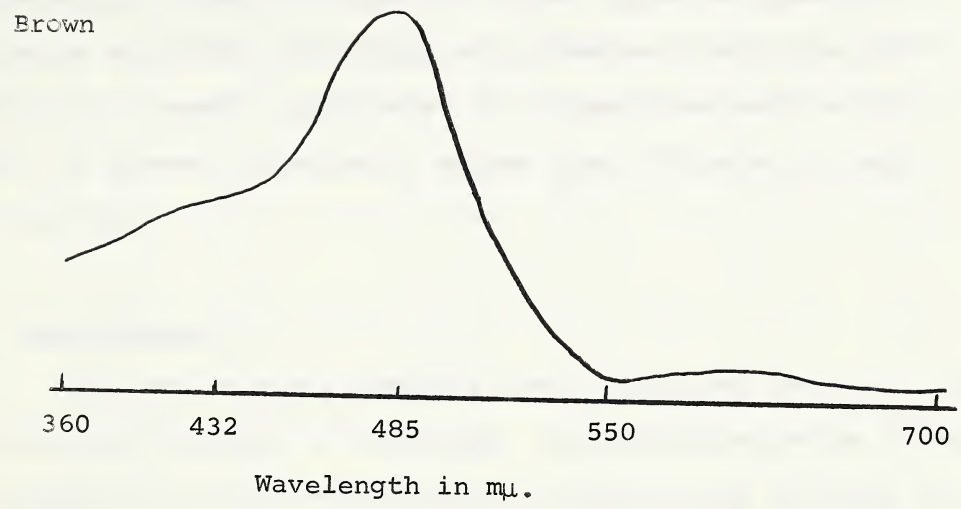
[a] Samples (45 mg. carotene plus 90 mg. diluent) were stored in clear gelatin capsules.

[b] Optical density of carotene in chloroform was determined at 466 mμ. Initial O.D. was .850.

[c] Wt. indicates the weight of the active material present and is expressed in mg. per capsule.

[d] Loss indicates the average loss in percent of the active material.

[e] For added convenience, O.D. values were expressed in terms of $E^{1\%}_{1cm.}$ (See Appendix Table III)



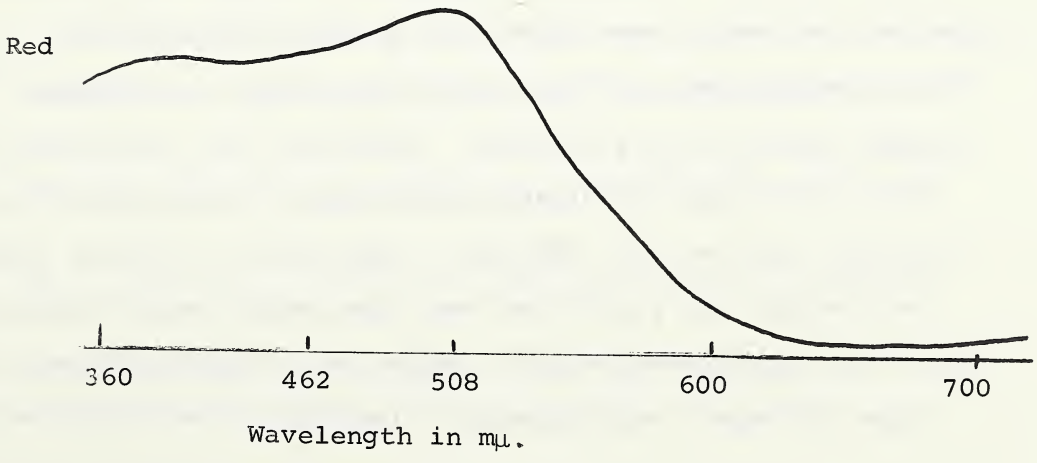


Fig. 6. Visible Absorption Spectra of Dyes used in Red and Brown Capsules

Capsules containing 120 mg. carotene were stored at room temperature in daylight. Weekly assays of carotene indicated no significant difference among the three series of capsules as shown in Table IV. Brown capsules appeared to increase stability initially, but subsequent oxidation proceeded at a normal rapid rate. No attempt was made in this study to prevent atmospheric oxygen from diffusing through the gelatin.

5. Antioxidants

Antioxidants are commonly used to prolong the stability of materials subject to oxidation. A literature review revealed the extensive use of antioxidants in stabilizing carotene in many systems, but apparently no studies had been performed on the use of antioxidants in stabilizing the carotenoid in the solid state, except in the form of forage and foodstuffs.

Antioxidants used in this study were chosen on the basis of acceptance in foods since they would be administered orally in combination with carotene. Canadian Food and Drugs Regulations* state that "a food shall contain not more than 0.01% propyl gallate, 0.005% NDGA, 0.01% BHT, of the final product", therefore these values were used as a basis for determining the concentrations to be tested. Since antioxidants are known to be effective in different concentrations, depending upon

---

* The Food and Drugs Act and Regulations, Department of National Health and Welfare, Canada.

TABLE IV

Effect of Light on Stability of Crystalline Beta-Carotene[a,e]

Time in Weeks	CAPSULE COLOR								
	Clear			Red			Brown		
	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]
2	.420	102.9	14.2	.428	104.8	12.4	.470	115.1	6.0
	.420	102.9		.428	104.8		.455	111.4	
	.420	102.9		.431	105.6		.458	112.2	
3	.385	94.3	20.8	.390	95.5	20.7	.390	95.5	20.6
	.388	95.0		.390	95.5		.390	95.5	
	.390	95.5		.385	94.3		.388	95.0	
4	.307	75.2	37.0	.305	74.7	37.5	.307	75.2	37.3
	.310	75.9		.304	74.5		.307	75.2	
	.310	75.3		.310	75.9		.307	75.2	
5	.240	58.7	50.8	.240	58.7	50.7	.250	61.2	50.2
	.243	59.4		.240	58.7		.238	58.2	
	.241	59.0		.245	60.0		.245	60.0	
6	.175	42.8	63.7	.180	44.1	63.2	.203	49.7	60.4
	.174	42.6		.181	44.3		.191	46.7	
	.184	45.0		.181	44.3		.189	46.3	

a Samples (120 mg. each) were stored at room temperature.

b Optical density of carotene in chloroform was determined at 466 mμ. Initial O.D. was .490.

c Wt. indicates the weight of the active material present and is expressed in mg. per capsule.

d Loss indicates the average loss in per cent of the active material.

e For added convenience, O.D. values were expressed in terms of $E^{1\%}_{1cm.}$ (See Appendix Table IV).

the system in which they are used, a ten-fold increase and a ten-fold decrease in concentration were also investigated.

To determine the most effective material in the present system, the antioxidants at the three concentrations were individually mixed with carotene and stored in clear gelatin capsules. To obtain the small amounts required, the antioxidants were diluted with lactose. The samples, stored at room temperature under normal light conditions, were assayed weekly for loss in potency. Results, shown in Tables V to VIII, indicated no significant difference in antioxidant effectiveness at the range of concentrations tested.

Because of the low toxicity and acceptability in high concentrations, the antioxidant effectiveness of alpha-tocopherol and ascorbic acid were studied. Ascorbic acid-carotene samples were treated as indicated above. The tocopherol-carotene samples, however, were stored in open one dram vials at an elevated temperature of 50°C. Ascorbic acid, added at 1% and 10% of the solid pigment offered no significant protection as shown in Table IX. At high concentrations, however, alpha-tocopherol appeared to be effective in increasing stability. Stability was seen to increase with increasing concentrations of tocopherol, as illustrated in Figure 7 and Table X.

Other antioxidants were not investigated at high concentrations because of the toxicity factor.

TABLE V

Effect of Nordihydroguaretic Acid on Stability of Crystalline Beta-Carotene[a]

Time in Weeks	CONTROL			NORDIHYDROGUARETIC ACID								
				Concentration 0.1%			Concentration 0.05%			Concentration 0.02%			Concentration 0.01%		
	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]
1	.480	117.5		.485	118.8		.481	117.8		.480	117.5		.473	115.8	
	.478	117.1	3.5	.480	117.5	1.7	.479	117.3	2.5	.480	117.5	2.2	.473	115.8	3.5
	.460	112.7		.479	117.3		.473	115.8		.479	117.3				
2	.420	102.9		.410	100.4		.410	100.4		.420	102.9		.410	100.4	
	.420	102,9	14.2	.415	101.6	15.7	.420	102.9	14.2	.420	102.9	14.2	.415	101.6	15.2
	.421	103.1		.415	101.6		.430	105.4		.420	102.9		.420	102.9	
3	.400	98.0		.388	95.0		.390	95.5		.395	96.7		.390	95.5	
	.392	96.0	19.4	.388	95.0	20.8	.389	95.3	20.5	.400	98.0	18.8	.390	95.5	20.4
	.392	96.0		.388	95.0								.390	95.5	

[a] Samples (120 mg. carotene with added antioxidant) were stored in clear gelatin capsules at room temperature.

[b] Optical density of carotene in chloroform was determined at 466 mμ. Initial O.D. was .490.

[c] Wt. indicates the weight of the active material present and is expressed in mg. per capsule.

[d] Loss indicates the average loss in per cent of the active material.

TABLE VI

Effect of Butylated Hydroxytoluene on Stability of Crystalline Beta-Carotene[a]

Time in Weeks	BUTYLATED HYDROXYTOLUENE											
	Concentration 0.1%			Concentration 0.05%			Concentration 0.02%			Concentration 0.01%		
	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]
1	.480	117.5		.481	117.8		.480	117.5		.465	113.9	
	.480	117.5	2.6	.480	117.5	2.1	.480	117.5	2.1	.455	111.4	
	.473	115.8		.478	117.1		.480	117.5		.455	111.4	6.5
2	.430	105.4		.410	100.4		.440	107.8		.420	102.9	
	.422	103.4	13.2	.420	102.9	14.9	.440	107.8	10.5	.420	102.9	14.2
	.422	103.4		.420	102.9		.435	106.5		.420	102.9	
3	.390	95.5		.382	93.6		.385	94.3		.380	93.1	
	.390	95.5	20.9	.382	93.6	22.0	.385	94.3	21.4	.383	93.8	22.1
	.383	93.8		.382	93.6		.385	94.3				

[a] Samples (120 mg. carotene with added antioxidant) were stored in clear gelatin capsules at room temperature.

[b] Optical density of carotene in chloroform was determined at 466 mμ. Initial O.D. was .490.

[c] Wt. indicates the weight of the active material present and is expressed in mg. per capsule.

[d] Loss indicates the average loss in per cent of the active material.

TABLE VII

Effect of Propyl Gallate on Stability of Crystalline Beta-Carotene[a]

Time in Weeks	PROPYL GALLATE											
	Concentration 0.1%			Concentration 0.05%			Concentration 0.02%			Concentration 0.01%		
	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]
	.482	118.0		.478	117.1		.475	116.3		.473	115.8	
1	.482	118.0	2.1	.479	117.3	3.1	.475	116.3	3.5	.473	115.8	2.6
	.476	116.5		.467	114.4		.475	116.3		.473	115.8	
	.431	105.6		.422	103.4		.421	103.1		.421	103.1	
2	.421	103.1	13.8	.415	101.6	14.7	.421	103.1	14.0	.421	103.1	14.5
	.419	102.6		.417	102.1		.423	103.5		.415	101.6	
	.391	95.8		.390	95.5		.389	95.3		.392	96.0	
3	.390	95.5	20.4	.385	94.3	21.1	.388	95.0	20.7	.389	95.3	20.5
	.389	95.3		.385	94.3		.388	95.0		.388	95.0	

[a] Samples (120 mg. carotene with added antioxidant) were stored in clear gelatin capsules at room temperature.

[b] Optical density of carotene in chloroform was determined at 466 mμ. Initial O.D. was .490.

[c] Wt. indicates the weight of the active material present and is expressed in mg. per capsule.

[d] Loss indicates the average loss in per cent of the active material.

TABLE VIII

Effect of Alpha-Tocopherol on Stability of Crystalline Beta-Carotene[a]

Time in Weeks	ALPHA-TOCOPHEROL											
	Concentration 0.1%			Concentration 0.05%			Concentration 0.02%			Concentration 0.01%		
	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]
	.480	117.5		.480	117.5		.472	115.6		.480	117.5	
1	.480	117.5	2.6	.478	117.1	2.5	.465	113.9	4.3	.475	116.3	2.6
	.473	115.8		.475	116.3		.455	111.4		.477	116.9	
	.450	110.2		.435	106.5		.425	104.1		.440	107.8	
2	.425	104.1	13.2	.430	105.4	12.0	.424	104.0	13.3	.440	107.8	10.8
	.425	104.1		.429	105.2		.424	104.0		.430	105.4	
	.400	98.0		.380	93.1		.388	95.0		.392	96.0	
3	.390	95.5	20.1	.388	95.0	21.3	.388	95.0	20.8	.388	95.0	20.6
	.385	94.3		.388	95.0		.388	95.0		.388	95.0	

[a] Samples (120 mg. carotene with added antioxidant) were stored in clear gelatin capsules at room temperature.

[b] Optical density of carotene in chloroform was determined at 466 mμ. Initial O.D. was .490.

[c] Wt. indicates the weight of the active material present and is expressed in mg. per capsule.

[d] Loss indicates the average loss in per cent of the active material.

TABLE IX

Effect of High Concentrations of Ascorbic Acid on Stability of Crystalline Beta-Carotene[a]

Time in Weeks	Control			1% Ascorbic Acid			10% Ascorbic Acid		
	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]
1	.475 .473	116.3 115.8	3.3	.473 .473	115.8 115.8	3.5	.475 .468	116.3 114.6	3.7
2	.415 .416	101.6 101.7	15.2	.410 .415	100.4 101.6	15.8	.420 .420	102.9 102.9	14.2
3	.380 .382	93.1 93.6	22.2	.383 .385	93.8 94.3	21.7	.381 .381	93.4 93.4	22.2

[a] Samples (120 mg. carotene with added antioxidant) were stored in clear gelatin capsules at room temperature.

[b] Optical density of carotene in chloroform was determined at 466 mμ. Initial O.D. was .490.

[c] Wt. indicates the weight of the active material present and is expressed in mg. per capsule.

[d] Loss indicates the average loss in per cent of the active material.

TABLE X

Effect of High Concentrations of α-Tocopherol on Stability of Crystalline Beta-Carotene[a, e]

Time in Days	Control			5% Tocopherol			10% Tocopherol			25% Tocopherol		
	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]
	.420	85.7		.425	86.7		.445	90.9		.445	90.9	
2	.421	85.9	14.2	.430	87.7	13.0	.446	91.0	9.0	.447	91.2	8.9
	.355	72.4		.408	83.3		.415	84.7		.425	86.7	
3	.362	73.9	26.9	.370	75.5	20.6	.415	84.7	15.3	.415	84.7	14.3
	.270	55.1		.310	63.2		.360	73.5		.390	79.6	
4	.270	55.1	44.9	.310	63.2	36.8	.360	73.5	26.5	.390	79.6	20.4
	.180	36.7		.210	42.8		.241	49.2		.290	59.2	
6	.180	36.7	63.3	.210	42.8	57.2	.240	49.0	50.9	.290	59.2	40.8

[a] Samples (100 mg.) were stored in open vials at 50°C.

[b] Optical density of carotene in chloroform was determined at 466 mμ. Initial O.D. was .490.

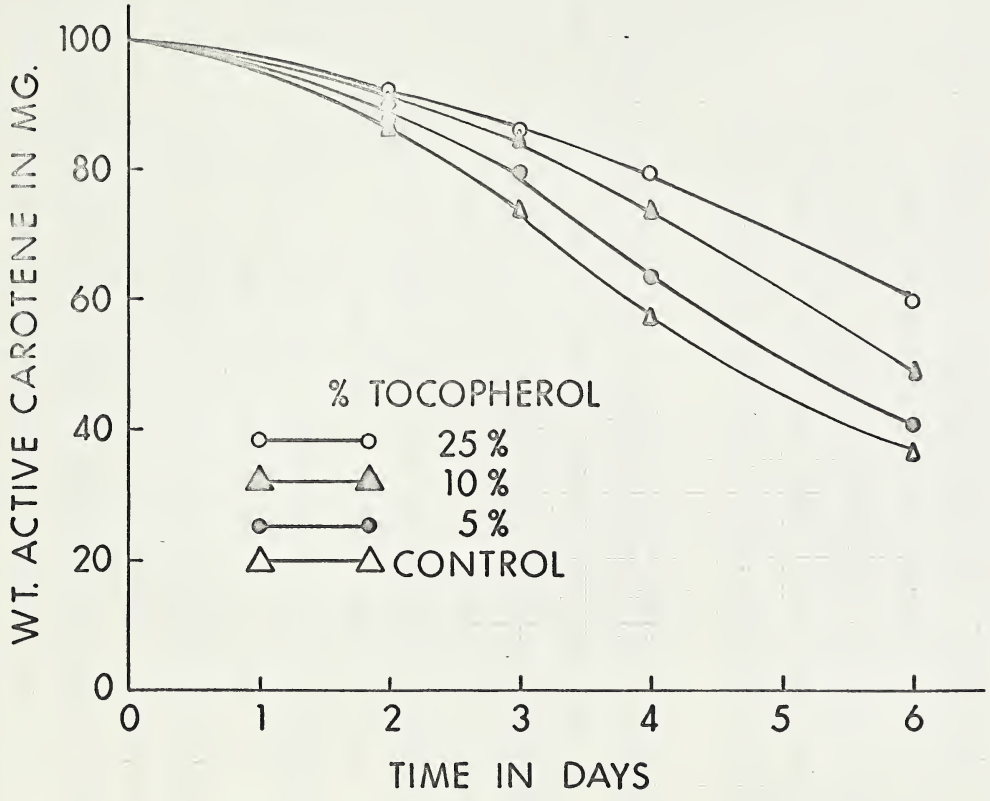
[c] Wt. indicates the weight of the active material present and is expressed in mg. per vial.

[d] Loss indicates the average loss in per cent of the active material.

[e] For added convenience, O.D. values were expressed in terms of $E^{1\%}_{1cm.}$ (See Appendix Table V)

Fig. 7

Effect of High Concentrations of Alpha-Tocopherol on Stability of Crystalline Beta-Carotene


WT. ACTIVE CAROTENE IN MG.
100
80
60
40
20
0
0
1
2
3
4
5
6
TIME IN DAYS
% TOCOPHEROL
25 %
10 %
5 %
CONTROL

6. Recrystallization

Since different commercial sources of beta-carotene exhibited variation in $E^{1\%}_{1cm.}$, and since oxidation proceeded at such a rapid rate, the possibility of an impurity being present, acting as a pro-oxidant, was considered. Therefore, efforts were made to purify the carotene samples by recrystallization. Fraps and Kemmerer found that recrystallization removed impurities in some commercial sources of carotene, thereby providing a purer, more stable product (83). Budowski and Bondi used recrystallization as a means of purifying carotene before it was subjected to oxidation studies (53).

In this work, carotene was recrystallized from a chloroform-methanol system. The recrystallized product was collected by gravity filtration and dried in a vacuum over calcium chloride. Recrystallization was found to be effective in purifying oxidzed carotene, i.e., a carotene sample of known potency was oxidized and then recrystallized. The extinction values proved to be identical before oxidation and after recrystallization. Repeated recrystallization failed to provide subsequent increases in potency, therefore it was concluded that one recrystallization was sufficient to remove any impurities. Subsequent recrystallization of freshly opened vials of "pure" carotene* failed to increase the $E^{1\%}_{1cm.}$, thereby indicating the absence of impurities.

Rates of oxidation of the recrystallized and unrecrystallized samples were compared at different temperatures: 37.5°C., ambient

---

* Carotene (100% beta) supplied by Distillation Products Ind.

temperature, and -10°C. While recrystallization was found to be effective in removing impurities and oxidized compounds, the product was found to oxidize more rapidly than the original material.

The data as given in Tables XI and XII and as presented graphically in Figure 8 indicate the degree of acceleration of the oxidative process as a result of recrystallization. It was felt that the rate of increase in degradation may have been due to the formation of much finer crystals in the purified products. If this assumption were true, it would result in a larger surface of carotene being exposed to the atmosphere with a corresponding increase in the rate of oxidation.

To determine whether this was indeed the case, examination of the pigment was made before and after recrystallization. Using a calibrated microscope, the original material was found to contain crystals ranging in size from 5 μ to 216 μ. Average diameter was estimated at approximately 72 μ. On the other hand, the recrystallized carotene was observed to be in the form of rod-shaped crystals so fine as to show active Brownian movement. Size of the particles varied over a narrow range with an estimated average length of 5 to 10 μ, thus confirming that a dramatic increase in surface area was obtained through recrystallization of carotene by the method used.

TABLE XI

Comparative Rates of Oxidation of Recrystallized[f] and Unrecrystallized Beta-Carotene[a,e]

37.5°C.								Ambient Temperature (25°C.)							
Unrecrystallized Control					Recrystallized				Control				Recrystallized		
Time in Weeks	O.D.[b]	Wt.[c]	Loss[d]	Time in Days	O.D.[b]	Wt.[c]	Loss[d]	Time in Weeks	O.D.[b]	Wt.[c]	Loss[d]		O.D.[b]	Wt.[c]	Loss[d]
1	.772	80.4		3	.720	75.0		1	.910	94.8			.680	70.7	
	.773	80.5	19.5		.720	75.0	25.0		.898	93.5	5.8		.681	70.8	29.2
2	.510	53.1		6	.408	42.6		2	.870	90.6			.382	39.8	
	.520	54.2	46.3		.406	42.3	57.6		.871	90.7	9.3		.382	39.8	60.2
4	.180	18.7		9	.212	22.0		4	.724	75.4			.005	0.52	
	.180	18.7	81.3		.208	21.6	78.2		.728	75.8	24.4		.004	0.41	99.5
5	.118	12.2		12	.110	11.5		6	.498	51.8					
	.114	11.8	88.0		.110	11.5	88.5		.510	53.1	47.5		-	-	-
6	.020	2.0						8	.237	24.7					
	.020	2.0	98.0	-	-	-	-		.237	24.7	75.3		-	-	-

a Samples (100 mg.) were stored in open vials.

b Optical density of carotene in chloroform was determined at 466 mμ. Initial O.D. was .960.

c Wt. indicates the weight of the active material present expressed in mg. per vial.

d Loss indicates the average loss of the active material in percent.

e For added convenience, O.D. values were expressed in terms of $E^{1\%}_{1cm.}$ (See Appendix Table VI)

f Carotene was recrystallized from a chloroform-methanol system.

TABLE XII

Comparative Rates of Oxidation of Recrystallized[f] and Unrecrystallized Beta-Carotene at -10°C.[a,e]

Time in Weeks	Recrystallized			Unrecrystallized (Control)		
	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]
1	.920 .924	95.8 96.2	4.0	.954 .956	99.3 99.5	0.6
2	.880 .880	91.6 91.6	8.4	.944 .945	98.2 98.3	1.7
4	.870 .850	90.6 88.5	10.6	.940 .950	97.9 98.9	1.6
6	.860 .860	89.6 89.6	10.6	.960 .940	100.0 97.9	1.1
8	.850 .844	88.5 87.9	11.8	.950 .950	98.9 98.9	1.1
12	.800 .800	83.3 83.3	16.7	.920 .910	95.8 94.7	4.7
16	.770 .750	80.2 78.1	20.8	.900 .900	93.7 93.7	6.3
24	.490 .491	51.0 51.1	48.9	.900 .904	93.7 94.1	6.1
32	.418 .417	43.5 43.3	56.6	.900 .900	93.7 93.7	6.3

[a] Samples (100 mg.) were stored in open vials.

[b] Optical density of carotene in chloroform was determined at 466 mμ. Initial O.D. was .960.

[c] Wt. indicates the weight of the active material present and is expressed in mg. per vial.

[d] Loss indicates the average loss in per cent of the active material.

[e] For added convenience, O.D. values were expressed in terms of $E_{1cm.}^{1\%}$ (See Appendix Table VII).

[f] Carotene was recrystallized from a chloroform-methanol system.

Fig. 8

Comparative Rates of Oxidation of Recrystallized and Unrecrystallized Beta-Carotene

●——● Unrecrystallized; stored at -10°C.

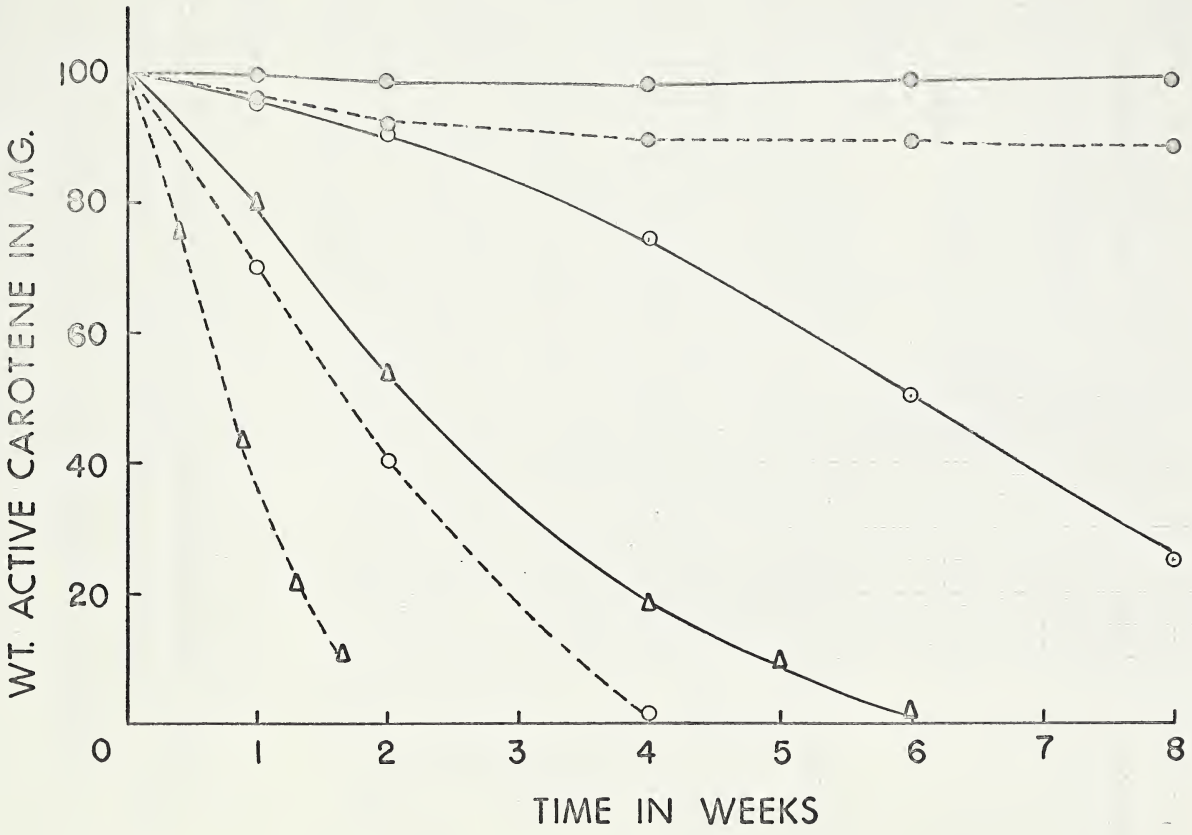
●----● Recrystallized; stored at -10°C.

o——o Unrecrystallized; stored at room temperature

o----o Recrystallized; stored at room temperature

Δ——Δ Unrecrystallized; stored at 37.5°C.

Δ----Δ Recrystallized; stored at 37.5°C.


WT. ACTIVE CAROTENE IN MG.
100
80
60
40
20
0
1
2
3
4
5
6
7
8
TIME IN WEEKS

B. Stability Studies on Selected Carotene Formulations

1. Solid Dosage Forms

a. Tablets

The following formula was used for all tablets prepared.

Basic Formula for 380 tablets

Beta-carotene	45.6 gm.
Lactose	87.4 gm.
Starch	16.0 gm.
Starch Paste (10%)	4.9 gm.
Dried Starch	4.2 gm.
Magnesium Stearate	1.4 gm.

The tablets were prepared by the Wet Granulation Method. Briefly, the procedure involved mixing the sieved powders with the binder, screening the wet mass through a #16 screen, drying the granulation at an indicated temperature of 50°C., rescreening the dried granules through the #16 screen to obtain a uniform granulation, and finally compressing the granules into tablets.* To insure tablets of uniform weight and potency, sufficient granulation was accurately weighed for each tablet and then compressed individually. Tablet hardness was found to be in the range of 9 to 11 kgm./sq.in.+

Assay Procedure

To determine the amount of active carotene per tablet, each tablet was subjected to the following procedures: a whole tablet was crushed and dissolved in chloroform. Insoluble

---

* Stokes Model Eureka (A3) Tablet Machine (F.J. Stokes Corp., Philadelphia, Pa.)

\+ Monsanto-Stokes Hardness Tester.

excipients were removed by filtration through a coarse sintered glass funnel. The filtrate and washings were collected and diluted to volume. Optical density values were then determined.

Preservation of Tablets

In an effort to determine whether tablet coating would be useful in maintaining tablet potency, it was decided to investigate the effect of polyethylene glycol 6000 (PEG 6000) as a coating material.

A sufficiently large batch of tablets was prepared by the method as indicated above. The wet granules were dried in a forced air oven at 50°C. for ninety minutes. After compression, one half of the tablets were reserved to serve as the control while the remainder were subjected to the coating procedure. In this study, the coating was applied in two steps. Tablets were first dipped by hand into a 25 per cent solution of PEG 6000 in alcohol. When this base coat had dried, the tablets were dipped into a 50 per cent solution of PEG 6000 in alcohol to seal any holes or defects which may have been present in the first coat (89).

Both the coated and uncoated tablets were placed in open vials to determine their stability. When exposed to air at room temperature, the tablets, as shown in Table XIII were seen to decompose exceptionally rapidly. The uncoated and coated tablets suffered the same degree of oxidative deterioration. Since this decomposition proceeded at a rate approximately twice that of the crystalline carotene, which served as a blank, it was suggested

TABLE XIII

Stability of Beta-Carotene Tablets (Coated with PEG 6000) Exposed to the Atmosphere[a]

Time in Days	Blank (Carotene Powder)			Control (Uncoated Tablets)			Coated Tablets		
	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]
7	.872	108.3		.867	134.6		.841	130.5	
	.872	108.3	9.7	.880	136.4	6.0	.810	125.7	10.0
				.837	130.0				
9	.849	105.5		.780	121.2		.705	109.4	
	.848	105.3	12.2	.740	114.9	17.1	.750	116.4	20.6
11	.820	102.5		.702	109.0		.705	109.4	
	.820	102.5	14.6	.720	111.8	22.4	.693	107.6	24.8
							.670	104.0	
13	.800	99.4		.590	91.5		.553	85.8	
	.799	99.4	17.2	.650	100.9	32.2	.562	87.2	38.9
				.625	97.0		.566	87.8	

[a] Stored at room temperature in open vials.

[b] Optical density of carotene in chloroform was determined at 466 mμ. Initial O.D. of blank was .966. Initial O.D. of tablets was .916.

[c] Wt. indicates the weight of active material present expressed in mg. Initial weight of blank was 120 mg. per vial. Initial weight of tablets was 142.2 mg., a value which represents the average of 7 tablets.

[d] Loss indicates the average loss of the active material in per cent.

that oxidation had been initiated during the drying of the granules.

Since the first batch of tablets appeared to be very unstable, a second batch was prepared with a reduction of the drying time to thirty minutes. The PEG film coat was not reconsidered as a coating as it was seen to offer a number of disadvantages. It appeared to have no control over rate of oxidation of the tablets. If the coating had had any effect, this would have been evident when compared with the uncoated tablets, even though oxidation had been initiated. Also, this coating did not provide a satisfactory appearance. As it was felt that clinical testing of this dosage form might be indicated, this material was discarded in favor of a more satisfactory coating formulation.

A literature search revealed the use of polyvinylpyrrolidone (PVP) and carnauba wax as coating materials.

A coating formulation using PVP, as presented in Drug Standards (90), was modified to suit the purposes of this study. A plasticizer was not employed, as it was considered unnecessary in this work. The PVP offered the advantage of good film forming and the additional wax deposit would provide a harder seal. A 10% PVP in 95% ethanol solution was used as the prime coat. The second coat consisted of carnauba wax in ether. This latter coat was applied twice.

Since these tablets were to be evaluated clinically, their disintegration time was investigated. Using the U.S.P. method*, studies were performed on the uncoated and coated tablets. Disintegration time was determined in Simulated Gastric Fluid maintained at 37°C.

---

* United States Pharmacopoeia, 17th Ed., p. 934.

TABLE XIV

Disintegration Times[a]

Uncoated Tablets	Tablets coated with PVP and carnauba wax
Time in seconds	Time in seconds
55	120
55	135
60	140
60	140
70	175
80	180

[a] Each value represents time required for one tablet to disintegrate completely.

The tablets, when exposed to air, oxidized at a rate comparable to that of the carotene powder. Although reduction of drying time improved the stability of the tablet by eliminating the rapid initiation of the reaction, it did not produce tablets which displayed any increase in stability over the powder. The PVP-carnauba wax coating was found to be ineffective in protecting the carotene from atmospheric oxygen, as illustrated in Table XV.

Since carotene was shown earlier to be relatively stable in a stagnant atmosphere, both uncoated and coated tablets were stored at selected temperatures in stoppered vials. Vials used were the 20 cc. serum type with a tight-fitting rubber closure as the seal. In general, all tablets remained stable for six months at 50°C., 37.5°C., and room temperature (Tables XVI,XVII,XVIII). However, low values were obtained in 3 instances.

TABLE XV

Stability of Beta-Carotene Tablets (Coated with PVP and Carnauba Wax) Exposed to the Atmosphere[a]

Time in Weeks	Blank (Carotene Powder)			Control (Uncoated Tablets)			Coated Tablets		
	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]
2	.435	112.7		.518	111.0		.490	105.0	
	.433	112.2	6.3	.506	108.4	9.0	.495	106.1	12.0
	.433	112.2		.506	108.4		.493	105.5	
3	.371	96.2		.455	97.5		.465	99.6	
	.355	92.0	21.7	.460	98.5	17.7	.485	103.9	15.2
	.355	92.0							
4	.312	80.7		.415	88.9		.392	84.0	
	.312	80.7	32.7	.418	89.6	24.8	.420	90.0	26.9
	.312	80.7		.420	90.0				
5	.240	62.2		.245	52.5		.265	56.8	
	.241	62.4	51.9	.245	52.5	54.6	.265	56.8	52.5
				.265	56.8				
6	.145	37.6		—	—		.190	40.7	
	.145	37.6	68.7	—	—	—	.190	40.7	65.7
8	—	—		.120	25.7		.129	27.7	
	—	—	—	.130	27.8	77.7	.131	27.9	76.7

[a] Stored at room temperature in open vials.

[b] Optical density of carotene in chloroform determined at 466 mμ. Initial O.D. of blank was.463. Initial O.D. of tablets was .554.

[c] Weight of active material present expressed in mg. Initial weight of blank was 120 mg. per vial. Initial weight of tablet was 118.7, a value which represents the average of 7 tablets.

[d] Average loss of active material in per cent.

TABLE XVI

Stability of Beta-Carotene Tablets Stored in Closed Vials[a]

at Room Temperature

Time in Weeks	Blank[b] (Carotene Powder)			Control (Uncoated Tablets)			Coated Tablets[c]		
	O.D.[d]	Wt.[e]	Average Wt.	O.D.[d]	wt.[e]	Average Wt.	O.D.[d]	Wt.[e]	Average Wt.
1	.459	119.0		.530	113.6		.491	105.2	
	.459	118.0	118.5	.511	109.5	111.6	.490	105.0	105.6
							.497	106.5	
2	.452	117.0		.518	111.0		.490	105.0	
	.455	118.0	117.5	.506	108.4	109.3	.495	106.1	105.5
				.506	108.4		.493	105.5	
3	.371	96.2		.470	100.6		.530	113.6	
	.355	92.0	94.1	.490	105.0	102.8	.491	105.2	109.4
				.480	102.9				
4	.312	80.7		.521	111.6		.483	103.5	
	.312	80.7	80.7	.500	107.0	109.3	.498	106.7	104.8
							.487	104.3	
5	.287	73.3					.504	108.0	
	.287	73.3	73.3	-	-	-	.520	111.4	109.7

TABLE XVI (Cont'd.)

Stability of Beta-Carotene Tablets Stored in Closed Vials[a]

at Room Temperature

Time in Weeks	Blank[b] (Carotene Powder)			Control (Uncoated Tablets)			Coated Tablets[c]		
	O.D.[d]	Wt.[e]	Average Wt.	O.D.[d]	Wt.[e]	Average Wt.	O.D.[d]	Wt.[e]	Average Wt.
6				.524	112.1		.510	109.3	
				.509	109.1	109.9	.480	102.9	106.1
				.507	108.6				
7				-	-	-	.490	105.0	
							.478	102.4	103.7
8				.483	103.5		.483	103.5	
				.473	101.4	102.5	.500	107.1	105.2
							.490	105.0	
12				.495	106.1		.500	107.1	
				.490	105.0	105.6	.492	105.3	106.1
							.490	105.0	
24				.520	111.4		.490	105.0	
						111.4	.520	111.4	108.2

[a] Rubber stoppered.

[b] Discontinued after fifth week due to extreme variability in results obtained.

[c] Coated with polyvinylpyrrolidone and carnauba wax.

[d] Optical density of carotene in chloroform was determined at 466 mμ. Initial O.D. of blank was .463. Initial O.D. of tablets was .554.

[e] Wt. indicates the weight of the active material present expressed as mg. Initial weight of blank was 120 mg. per vial. Initial amount of carotene per tablet was 118.7 mg., a value which represents the average of 7 tablets.

TABLE XVII

Stability of Beta-Carotene Tablets Stored in Closed Vials[a] at 37.5°C.

Time in Weeks	Blank[b] (Carotene Powder)			Control (Uncoated Tablets)			Coated Tablets[c]		
	O.D.[d]	Wt.[e]	Average Wt.	O.D.[d]	Wt.[e]	Average Wt.	O.D.[d]	Wt.[e]	Average Wt.
1	.723	93.7		.482	103.3		.500	107.1	
	.720	93.3	93.5	.492	105.4	106.9	.520	111.4	108.0
				.523	112.1		.492	105.4	
2	.663	85.8		.520	111.4		.480	102.9	
	.660	85.5	85.7	.500	107.1	109.3	.481	103.1	103.7
				.510	109.3		.490	105.0	
3	-	-	-	.528	113.1		.505	108.2	
				.523	112.1	112.2	.512	109.7	109.3
				.520	111.4		.513	109.9	
4	.500	64.8		.516	110.6		.415	88.9	
	.499	64.7	64.8	.500	107.1	109.9	.414	88.8	90.0
				.523	112.1		.431	92.3	
5	.468	60.6					.492	105.4	
	.465	60.3	60.4	-	-	-	.480	102.9	104.2

TABLE XVII (Cont'd.)

Stability of Beta-Carotene Tablets Stored in Closed Vials[a] at 37.5°C.

Time in Weeks	Blank[b] (Carotene Powder)			Control (Uncoated Tablets)			Coated Tablets[c]		
	O.D.[d]	Wt.[e]	Average Wt.	O.D.[d]	Wt.[e]	Average Wt.	O.D.[d]	Wt.[e]	Average Wt.
6				.440	94.3		.530	113.6	
				.448	96.0	95.2	.530	113.6	111.2
							.496	106.3	
7							.522	111.8	
				-	-	-	.490	105.0	106.8
							.483	103.5	
8				.520	111.4		.485	104.0	
				.498	106.7	108.1	.493	105.5	105.0
				.495	106.1		.493	105.5	
12				.480	102.9		.525	112.5	
				.495	106.1	107.5	.493	105.5	107.7
				.530	113.5		.490	105.0	
24				.510	109.3	109.3	-	-	-

[a] Rubber stoppered.

[b] Discontinued after fifth week due to extreme variability in results obtained.

[c] Coated with polyvinylpyrrolidone and carnauba wax.

[d] Optical density of carotene in chloroform was determined at 466 mμ. Initial O.D. of blank was .916. Initial O.D. of tablets was .554.

[e] Weight of active material present expressed as mg. Initial weight of blank was 120 mg. per vial. Initial amount of carotene per tablet was 118.7 mg., a value which represents the average of 7 tablets.

TABLE XVIII

Stability of Beta-Carotene Tablets Stored in Closed Vials[a] at 50°C.

Time in Weeks	Blank[b] (Carotene Powder)			Control (Uncoated Tablets)			Coated Tablets[c]		
	O.D.[d]	Wt.[c]	Average Wt.	O.D.[d]	Wt.[c]	Average Wt.	O.D.[d]	Wt.[c]	Average Wt.
1	.544	70.5		.509	109.1		.491	105.2	
	.550	71.3	70.9	.480	102.9	104.9	.480	102.9	104.1
	.548	71.0		.479	102.6				
2	.603	78.1		.510	109.3		.444	95.1	
	.606	78.5	78.3	.503	107.8	108.6	.350	75.0	87.4
							.433	92.8	
3	.520	67.4		.497	106.5		.512	109.7	
	.510	66.1	66.8	.526	112.7	109.6	.513	109.9	109.8
4	.375	48.6		.515	110.4		.540	115.7	
	.374	48.5	48.6	.490	105.0	107.7	.546	117.0	113.1
				.502	107.6		.498	106.7	
5	.366	47.4					.498	106.7	
	.364	47.2	47.3	-	-	-	.513	109.9	108.3

TABLE XVIII (Cont'd.)

Stability of Beta-Carotene Tablets Stored in Closed Vials[a] at 50°C.

Time in Weeks	Blank[b] (Carotene Powder)			Control (Uncoated Tablets)			Coated Tablets[c]		
	O.D.[d]	Wt.[e]	Average Wt.	O.D.[d]	Wt.[e]	Average Wt.	O.D.[d]	Wt.[e]	Average Wt.
6				.465	99.6		.484	103.8	
				.390	83.6	93.6	.500	107.1	106.9
				.455	97.6		.513	109.9	
7							.478	102.4	
				-	-	-	.490	105.0	105.1
							.503	107.8	
8				.498	106.7		.495	106.1	
				.509	109.1	108.3	.515	110.4	106.5
				.510	109.3		.480	102.9	
12				.490	105.0		.485	103.8	
				.505	108.2	107.5	.487	104.4	104.0
				.510	109.3		.485	103.8	
24				.500	107.1	107.1	-	-	-

[a] Rubber stoppered.

[b] Discontinued after fifth week due to extreme variability in results obtained.

[c] Coated with polyvinylpyrrolidone and carnauba wax.

[d] Optical density of carotene in chloroform was determined at 466 mμ. Initial O.D. of blank was .916. Initial O.D. of tablets was .554.

[e] Weights of active material present expressed as mg. Initial weight of blank was 120 mg. per vial. Initial amount of carotene per tablet was 118.7 mg., a value which represents the average of 7 tablets.

All tablets experienced an initial decomposition of approximately 10% after which no significant loss of the active ingredient occurred within the six month period. Apparently, due to the limited supply of oxygen in the vial, the reaction ceased when available oxygen became too low.

After a short period, the carotene powder which served as a blank began to yield varying results. These results were not readily explainable since all of the tablets with the exception of the three lots mentioned above, remained stable in the same type of containers and under identical conditions.

2. Liquid Dosage Forms

Because carotene is very unstable in the solid state and stabilization appeared to be very difficult in this form, the preparation of a liquid dosage form containing carotene was investigated. Only fats and oils were considered as vehicles, as they were known to enhance carotene absorption. Initial consideration of a liquid dosage form revealed at least two disadvantages associated with such a preparation. These included the problems of reduced accuracy and limited solubility. Consultation with medical staff at the University of Alberta Hospital indicated that an error of $\pm$ 20 per cent could be tolerated on the basis of a 120 mg. dose. Thus the importance of this factor was diminished and possibilities for this dosage form were increased. Limited solubility of carotene in oils proved to be the major problem, since a large amount of carotene would be required in a relatively small volume of oil.

Available vegetable oils were studied to determine the vehicle which offered the best protective action for carotene. Accelerated testing was employed using a small quantity of carotene. The carotene was weighed into 20 cc. serum vials containing one ml. of oil and the open vials were then stored at 50°C. They were assayed daily for carotene content. Crystalline carotene was used as the control and each oil was carried as a blank. Results obtained, as shown in Table XIX and Figure 9, were in agreement with published data indicating that corn oil offers less protection than cottonseed oil (62). The olive and cottonseed oils were considered comparable in protecting the carotene.

a. Emulsions

It was felt that a suspension of crystalline carotene in the oil phase of an emulsion should provide a relatively stable preparation since the pigment would be offered some protection from atmospheric conditions. A preliminary study on carotene in five ml. of cottonseed oil emulsion stored in closed vials at room temperature indicated stability for at least a three month period.

In considering cottonseed and olive oils for use in preparing the proposed emulsions, the olive oil was selected because of its higher viscosity and greater degree of saturation as indicated by a lower iodine number.

Since preliminary testing indicated that maximum stability of an olive oil-water emulsion was obtained at an HLB (Hydrophile-Lipophile Balance) of 8.3, the following general formula was prepared to conform

TABLE XIX

Stability of Beta-Carotene in Selected Vegetable Oils at 50°C.[a,e]

Time in Hours	Vegetable Oil											
	Control			Corn Oil			Cottonseed Oil			Olive Oil		
	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]
24	.432	17.7		.460	18.8		.480	19.6		.480	19.6	
	.432	17.7	11.5	.460	18.8	6.0	.480	19.6	2.5	.480	19.6	2.0
				.460	18.8		.474	19.4		.480	19.6	
48	.354	14.8		.450	18.5		.450	18.5		.458	18.8	
	.354	14.8	26.0	.450	18.5	8.0	.450	18.5	7.5	.453	18.6	7.0
				.444	18.2		.448	18.4		.448	18.4	
72	.240	10.0		.380	15.6		.438	18.0		.405	16.7	
	.238	9.9	50.0	.375	15.5	23.0	.433	17.8	11.0	.405	16.7	16.5
				.370	15.2		.417	17.2		.405	16.7	
120	.120	5.1		.240	10.0		.327	13.5		.318	13.1	
	.120	5.1	74.5	.240	10.0	47.5	.320	13.2	35.0	.312	12.9	36.0
				.280	11.6		.300	12.4		.300	12.4	
168	.073	3.2		.154	6.6		.200	8.4		.202	8.5	
	.073	3.2	84.0	.167	7.1	65.0	.203	8.5	57.5	.202	8.5	58.0
				.171	7.2		.210	8.7		.196	8.2	

[a] Samples (20 mg. carotene per ml. oil) stored in open vials.

[b] Optical density of carotene in chloroform determined at 466 mμ. Initial O.D. was .490.

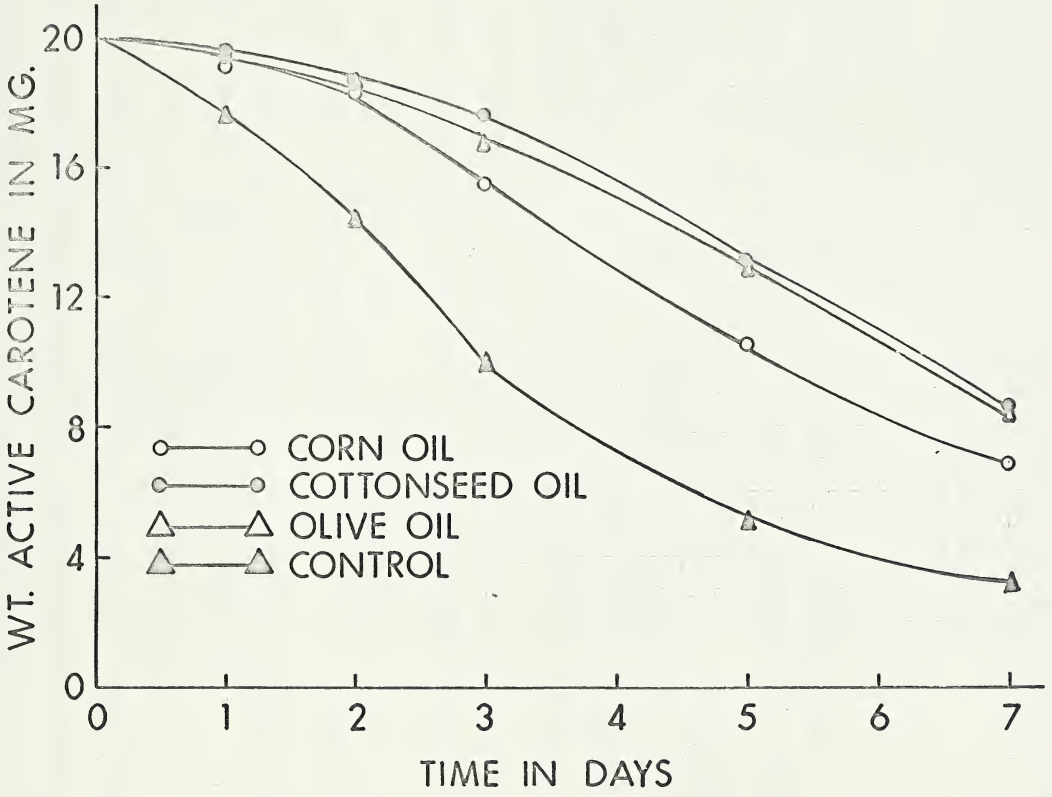
[c] Weight of active material present expressed in mg. per ml. oil.

[d] Average loss of active material in per cent.

[e] For added convenience, O.D. values were expressed in terms of $E^{1\%}_{1cm.}$ (See Appendix Table VIII)

Fig. 9

Stability of Beta-Carotene in Selected Vegetable Oils at 50°C.


WT. ACTIVE CAROTENE IN MG.
20
16
12
8
4
0
0
1
2
3
4
5
6
7
TIME IN DAYS
CORN OIL
COTTONSEED OIL
OLIVE OIL
CONTROL

to this value:

Olive oil	46.25 ml.
Distilled water	46.25 ml.
Span 60*, Tween 60+ Blend	5.00 gm.
Beta-carotene	2.50 gm.

Using a laboratory homogenizer, 48 fl. oz. of the above emulsion was prepared. Aliquots were withdrawn and stored in individual eight dram vials for shelf-life testing.

Assay Procedure

Theoretically five ml. of emulsion contained 125 mg. carotene. Separation and extraction of the carotene in this quantity of emulsion was accomplished by means of a separatory funnel. A brine solution was used to break the emulsion. The carotene was extracted with four 20 ml. portions of chloroform. The emulgents also entered the organic phase but were mechanically removed by filtration through a cotton plug. The chloroform extract was diluted to 100 ml. and appropriate dilutions of this solution were made later. Concentrations of carotene were determined spectrophotometrically. Olive oil in chloroform (in a 1 in 2000 dilution) served as a blank to compensate for olive oil which was also extracted by the solvent.

Assays of numerous five ml. samples showed varying results. It was not possible to duplicate results. Mechanical separation of the carotene by filtration did not prove practical. Neither the emulgents nor the olive oil present acted as

---

* Span 60. Trade name - sorbitan monostearate.

\+ Tween 60. Trade name - polyoxyethylene sorbitan monostearate (Atlas Chemical Industries, Inc., Brantford, Ontario).

interferants in the O.D. readings at the maxima involved at the concentrations used, as determined by a spectrophotometric scan of the material in chloroform in the visible region. The wide range of results obtained in the five ml. samples of carotene emulsion was attributed to a non-uniform dispersion of carotene particles throughout the emulsion.

Particle size was visibly non-homogeneous in a freshly-prepared emulsion. The emulsion was therefore processed through a colloid mill to reduce particle size. The resulting product was visibly thinner and particles displayed a tendency to float and agglomerate. Due to the difficulty encountered in obtaining uniform and accurate doses, the emulsion-type dosage form was considered not feasible for clinical purposes and further studies on this method were discontinued.

Since an emulsion was not acceptable as a vehicle, a less complex system was considered. As a result, a single phase vehicle was investigated.

b. Suspensions

Individual doses containing 100 mg. beta-carotene per five ml. cottonseed oil were prepared and assayed for stability.

Assay Procedure

Method of assay involved a simple technique in which the entire suspension was dissolved in chloroform and the amount of carotene present was then determined spectrophotometrically. Bickoff et al (82) have stated that measurement of stability by this method may give false values as oxidation products

formed may affect O.D. readings with the result that high values may be obtained. However, chromatographic analysis of the suspensions, stored for six weeks, showed no colored oxidation products which would interfere with the values obtained. Columns employed were prepared from alumina and from magnesium oxide. Thus it was concluded that O.D. values obtained here were due to the amount of carotene present, and not to any interferant.

Stability was determined on samples continually exposed to the air, samples stored in closed vials, and samples stored under nitrogen*. Results are presented in Table XX. Storage was arranged at room temperature in diffuse light.

Stability studies performed here showed very little or no decrease in potency after six weeks. Studies were not continued for a longer period as time did not permit. As a result, total shelf-life of the system was not determined.

Since the medium was not sufficiently viscous to suspend the carotene, a uniform dose would not be obtained from a larger volume. The addition of more saturated, and therefore more viscous oils, such as coconut oil, failed to increase viscosity sufficiently. Addition of glycerol monostearate provided a good suspending medium. Glycerol monostearate, 2%, was added to the oil and heated until complete solution occurred. Carotene was added to the cooled solution.

---

* Nitrogen values were carried as a matter of interest only and are therefore not reported.

TABLE XX

Stability of Beta-Carotene in Cottonseed Oil at Room Temperature[a,e]

Time in Weeks	Suspension in Sealed Container			Suspension Exposed to Air		
	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]
1	.488	99.6		.480	98.0	
	.490	100.0	0.2	.472	96.3	1.9
	.489	99.8		.490	100.0	
2	.475	97.0		.480	98.0	
	.488	99.6	1.3	.480	98.0	1.3
	.488	99.6		.490	100.0	
3	.485	99.0		.465	95.0	
	.480	98.0	1.0	.480	98.0	3.3
	.490	100.0		.475	97.0	
4	.463	94.5		.490	100.0	
	.480	98.0	2.8	.480	98.0	2.5
	.485	99.0		.463	94.5	
6	.480	98.0		.460	94.0	
	.468	95.5	4.8	.462	94.4	5.9
	.455	92.0		.460	94.0	

[a] Cottonseed oil was carried as a blank. Crystalline carotene in closed containers, and carotene exposed to the air served as controls. Values obtained were previously reported (See Table II).

[b] Optical density of carotene in chloroform was determined at 466 mμ. Initial O.D. was .490.

[c] Weight of active material expressed as mg. per 5 ml. oil. Initial weight was 100 mg. per 5 ml. oil.

[d] Average loss of active material in per cent.

[e] For added convenience, O.D. was expressed in terms of $E^{1\%}_{1cm.}$ (See Appendix Table IX)

Stabilization

An aspect of carotene stability which has been studied extensively is stabilization in oils with the aid of antioxidants. Four common antioxidants were investigated in this laboratory. The effectiveness of these substances as stabilizers for carotene was determined by accelerated testing at 50°C. Each antioxidant was incorporated at the concentration suggested as the maximum in the Canadian Food and Drugs Regulations. A second series containing ten times this amount was also carried in parallel in the event that the suggested concentrations were ineffective. Antioxidants investigated included alpha-tocopherol (0.1% and 0.01%), NDGA (0.005% and 0.05%), propyl gallate (0.1% and 0.01%), and BHT (0.1% and 0.01%). Samples were assayed daily spectrophotometrically. No significant difference in antioxidant effectiveness was observed, as seen in Table XXII. After 96 hours, acceptable agreement among replicates could not be obtained. However, 25% oxidation was shown to have occurred at 96 hours without evidence of antioxidant effectiveness, therefore the study was terminated and it was concluded that the antioxidants tested offered no significant protection in the oils where a large quantity of carotene was involved.

TABLE XXII

Effect of Antioxidants on Stability of Beta-Carotene in Cottonseed Oil at 50°C.[a]

Time in Hours	Control			Propyl Gallate						α-Tocopherol					
				Conc. .01%			Conc. .1%			Conc. .01%			Conc. .1%		
	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]
48	.440	18.1		.450	18.4		.451	18.5		.440	18.1		.450	18.4	
	.440	18.1	9.5	.440	18.1	9.5	.440	18.1	8.5	.440	18.1	9.0	.450	18.4	8.5
	.443	18.2		.430	17.7					.448	18.4		.440	18.1	
72	.432	17.8		.420	17.3		.423	17.4		.423	17.4		.423	17.4	
	.421	17.3	12.5	.417	17.2	14.0	.424	17.4	13.0	.421	17.3	13.5	.424	17.2	13.5
	.420	17.3		.417	17.2					.421	17.3		.424	17.2	
96	.373	15.4		.372	15.3		.387	15.9		.380	15.7		.373	15.4	
	.374	15.4	22.5	.360	14.9	25.0	.378	15.6	21.5	.374	15.4	22.5	.374	15.4	23.5
	.375	15.5		.360	14.9		.378	15.6		.374	15.4		.369	15.2	

TABLE XXII (Cont'd.)

Effect of Antioxidants on Stability of Beta-Carotene in Cottonseed Oil at 50°C.[a]

Time in Hours	Butylated Hydroxytoluene						Nordihydroguaretic Acid					
	Conc. .01%			Conc. .1%			Conc. .005%			Conc. .05%		
	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]	O.D.[b]	Wt.[c]	Loss[d]
48	.430	17.7		.451	18.5		.440	18.1		.440	18.1	
	.428	17.6	11.0	.435	17.9	9.0	.440	18.1	10.5	.430	17.7	11.0
	.443	18.2					.425	17.6		.430	17.7	
72	.415	17.1		.418	17.2		.426	17.5		.420	17.3	
	.414	17.1	14.0	.420	17.3	13.5	.426	17.5	13.0	.417	17.2	14.0
	.420	17.3		.390	16.0		.420	17.3		.417	17.2	
96	.379	15.7		.381	15.8		.380	15.7		.382	15.8	
	.376	15.5	20.5	.379	15.7	21.5	.380	15.7	21.5	.380	15.7	22.0
	.400	16.4		.379	15.7					.373	15.4	

[a] Samples stored in open vials.

[b] Optical density of carotene in chlorofrom was determined at 466 mμ. Initial O.D. was .488.

[c] Weight of active material present expressed in mg.per ml. oil. Initial weight was 20 mg. per ml. oil.

[d] Average loss of active material in per cent.

C. Clinical Evaluation of Selected Dosage Forms

Since carotene is a fat-soluble pigment, estimations of serum levels can be taken as a measure of fat absorption. Low carotene levels are found in either undernourished patients or those with malabsorption syndromes. To differentiate between the two, an oral loading dose of carotene dispersed in fat is administered to the patient. If the patient does not have a significant increase in serum carotene in 24 hours, this is usually considered to be an indication of malabsorption. The patient is required to fast for eight hours prior to the administration of the test dose. At this time, usually in the morning, two tubes of fasting blood are drawn. The carotene is then given to the patient. Clotted blood specimens are drawn at 2:00 P.M., 5:00 P.M., 9:00 P.M., and 24 hours. The 24 hour specimen is a fasting sample. In some instances, further sampling is required so that fasting specimens at 48 and 72 hours are taken. The above procedure was observed with all subjects studied, with slight variation in the time of collection of blood samples when necessitated by hospital routine.

Assay Procedure

The assay of carotene in the blood serum is relatively simple, and is based on the principle that the fat-soluble pigment is extractable by organic solvents, and its concentration can be determined spectrophotometrically: two ml. volumes of serum were denatured by the addition of an equal volume of 95% ethanol. Three ml. of petroleum ether was added, and the system was shaken for ten minutes. The emulsion was broken by centrifugation and the petroleum ether layer removed for spectrophoto-

metric analysis. The optical density of the solution was read at 455 mμ*. The O.D. values were converted to serum carotene content as follows:

$$\text{Carotene (mcg./100ml.)} = \frac{\text{O.D.(test)}}{\text{O.D.(standard)}} \times \text{Conc.Std.per ml.} \times \frac{100}{2}$$

It was necessary to insure that the carotene remained stable in the serum until read. This was accomplished by refrigeration of the serum sample. A standard carotene solution and a "pool" serum sample were employed as reference standards.

The dosage forms to be investigated were administered to volunteer hospital patients and laboratory staff. Patients with normal gastro-intestinal tracts were chosen in consultation with medical staff. The majority of subjects were males, ranging in age from 22 to 82 years.

The criterion of this test was based on a blood carotene rise of more than 15 mcg. per cent. The medical staff at the University Hospital considered any lower value as indicative of malabsorption.

To obtain data to indicate the reliability of the test as presently employed, and to serve as a reference when evaluating the selected dosage forms, ten normal subjects were given 120 mg. carotene with 20 gm. butter. Subsequently, six known malabsorbers were given the same test dose. Results, as presented in Tables XXII and XXIII, indicated a significant rise in all normals while flat curves were obtained with the malabsorbers. A

---

* Beckman Model B spectrophotometer

TABLE XXII

Carotene Tolerance in Normal Subjects After a Loading Dose of Beta-Carotene in Butter[a]

Subject	Serum Carotene in mcg/100 ml. Time in Hours					Max. increase in mcg. percent
	0	4	8	12	24	
L.L.	78	79	93	96	89	18
T.F.	71	86	96	102	108	37
J.A.	107	117	139	118	127	32
M.A.	91	134	103	102	114	43
A.N.	97	97	98	102	128	31
S.M.	108	142	123	117	169	61
H.H.	77	89	120	122	117	45
N.M.	86	103	108	127	133	47
P.N.	64	98	74	77	89	34
T.C.	44	61	64	61	64	20

[a] Subjects received 120 mg. carotene in 20 gm. butter on a slice of bread.

TABLE XXIII

Carotene Tolerance in Patients with Malabsorption Syndromes After a Loading Dose of Beta-Carotene in Butter[a]

Subject	Serum Carotene in mcg/100 ml. Time in Hours					Max. increase in mcg. percent
	0	4	8	12	24	
M.M.	52	53	50	52	51	1
H.R.	13	14	13	14	17	4
D.D.	49	51	49	49	52	3
S.W.	61	64	68	59	66	7
J.J.	13	14	15	16	17	4
F.A.	12	12	12	12	11	0

[a] Subject received 120 mg. carotene in 20 gm. butter on a slice of bread.

typical carotene tolerance curve is presented in Figure 10 illustrating the rise in the normal patient and the flat curve displayed in the abnormal patient. It should be noted that although the Carotene Tolerance Test is performed on patients with low fasting serum carotenes, the majority of patients studied here had fasting levels within the normal range (70 - 282 mcgm./100 ml.). It was observed that some "normal" patients with high initial carotene levels failed to display an increase in serum carotene after administration. This was probably due to the fact that body stores of carotene were so high, that additional amounts were not reflected in the blood.

In this work, clinical studies were initiated with the tablet dosage form. One tablet was administered with water to each of six patients who had been diagnosed as normal. A seventh patient received a tablet with two bile salt capsules since bile salts had been claimed to enhance absorption (27). All subjects displayed flat curves, therefore it was concluded that the carotene was not absorbed. Since negative results were obtained, the possibility of using a fat to enhance the absorption of the tablets was investigated. Five normals were each given a tablet with fifteen ml. fat emulsion*. Two patients showed a normal rise in serum carotene. The remaining three patients, however, exhibited flat curves.

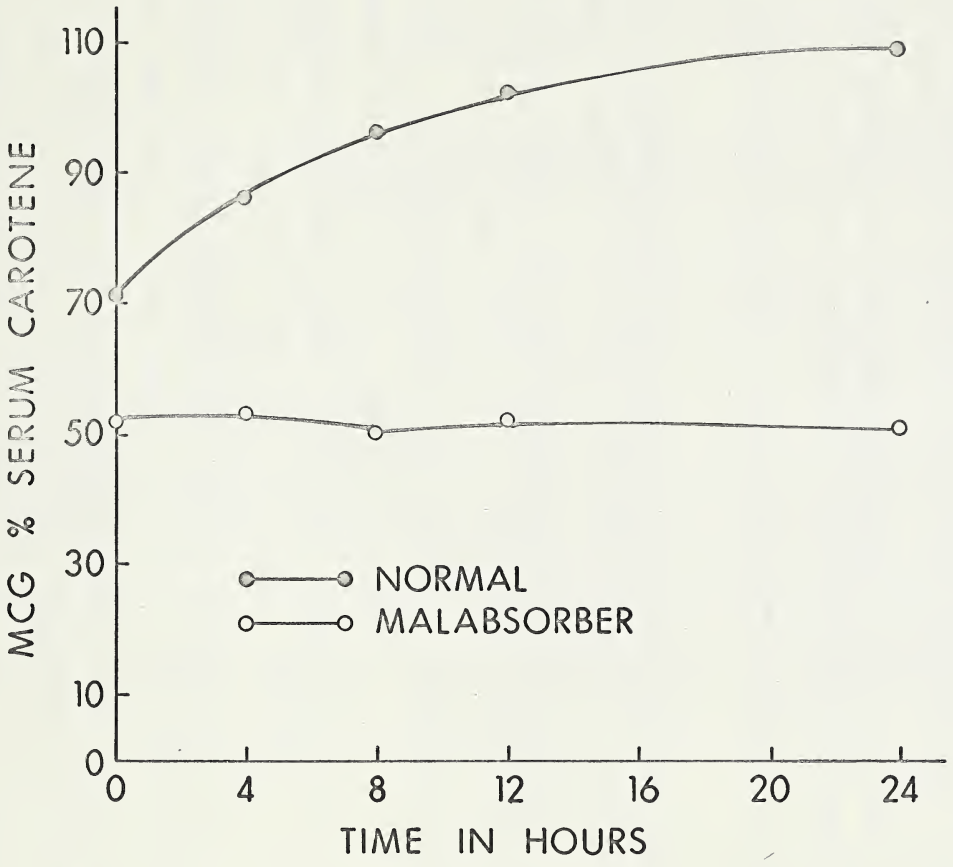
Nine patients were then given the tablet with twenty grams of butter, the amount administered in the regular test.

---

* Lipomul Oral (Upjohn Co., Toronto, Ontario)

Fig. 10

Typical Carotene Tolerance Curves


MCG % SERUM CAROTENE
110
90
70
50
30
10
0
0
4
8
12
16
20
24
TIME IN HOURS
NORMAL
MALABSORBER

Results, illustrated in Table XXIV, were varied, hence were considered unreliable. Four of the patients displayed flat curves while five showed very definite increases. Two of the patients with whom flat curves were obtained, had initial low carotene levels. Thus they may have been malabsorbers, although this was doubtful since their histories revealed no evidence of gastro-intestinal malfunction. The test could not be repeated on these patients as they were discharged from the hospital. Studies on the tablet dosage form were discontinued because not all of the normal patients tested displayed a normal tolerance curve.

In a subsequent patient, five ml. cottonseed oil suspension promoted absorption of 120 mg. carotene to give a significant rise in the serum. Further studies on two other patients did not produce the same effect, as no absorption of the drug was observed. In another patient, an emulsion containing 2.5 ml. cottonseed oil also gave a significant rise.

Carotene has been shown to be more stable in cocoa butter than in less saturated fats and oils (76). Therefore, as a potentially stable formulation, 120 mg. carotene in 2 gm. cocoa butter were given to two normal patients. The serum carotene gave a significant rise in one patient but a flat curve was obtained with a second. Four patients, diagnosed as normals, were further tested with this dosage form, but all displayed flat curves. No conclusion could be drawn from this series, as these patients had initial serum carotene levels below 70 mcg. per cent, again suggesting that these "normals" may have been undiagnosed malabsorbers.

TABLE XXIV

Carotene Tolerance in Normal Subjects After Administration of a Beta-Carotene Tablet[a] in Butter[b]

Subject	Serum Carotene in mcg/100 ml. Time in Hours					Max. increase in mcg. percent
	0	4	8	12	24	
P.J.	129	131	147	137	147	18
R.J.	69	75	79	77	79	flat
C.B.	72	64	67	62	69	flat
W.P.	122	117	121	123	119	flat
V.K.	165	164	168	183	202	37
C.D.	59	86	77	79	72	27
B.H.	152	177	187	187	202	50
S.P.	144	150	155	168	143	24
T.B.	77	74	76	69	76	flat

[a] Weight of carotene per tablet averaged 109.0 mg.

[b] Subjects received tablet with 20 gm. butter.

A saturated fat has been claimed to offer better protection of carotene than an unsaturated fat (53). A hydrogenated vegetable oil was considered as a promising vehicle. However, administration of 120 mg. carotene in a homogeneous mixture with a commercial product* to two normals produced flat curves. On this basis further tests with this vehicle were discontinued.

Thompson showed that particle size significantly affected absorption of carotene in cows (24). In his study, micropulverization was used to obtain particle size reduction. Since this method was not possible with the laboratory facilities available, recrystallization was used as a means of achieving the same end. Recrystallization was performed under nitrogen because of the sensitivity to oxidation. The standard dose of carotene, dispersed in fifteen ml. Lipomul Oral gave a very significant rise in serum carotene. However, since emulsions were considered to be a physically unacceptable dosage form, encountering patient resistance because of non-uniform dispersion of the pigment particles, they were not investigated further clinically.

In considering other possible carriers known to contain a significant amount of fat, a supply of solid milk chocolate was purchased. A portion of this was melted and levigated with 120 mg. carotene to form a uniform dispersion. This mixture was then poured into a suppository mold and chilled until solid. When administered later to a normal patient, a flat curve was obtained. Further testing was discontinued.

---

* Crisco - Trade name (Proctor and Gamble, Toronto, Ontario)

Since a clinically useful, stable pharmaceutical preparation had not been achieved, commercial food color concentrates were next investigated as possible stable dosage forms. Water dispersible beadlets of carotene were claimed to possess very good stability when exposed to air (86). Since a stable product of this type would be desirable on a practical basis, it was investigated clinically. Two groups of patients were selected for this purpose. One group received the beadlets in orange juice together with 20 gm. butter on a slice of bread while the other group received the beadlets in orange juice alone. Results are illustrated in Tables XXV and XXVI. When given with butter, the dispersion of carotene in orange juice, proved to be an effective method of administering the drug in 100 per cent of the cases. Two known malabsorbers were also given this preparation. A flat curve was obtained in both. When administered without butter, the carotene in orange juice was absorbed in four of the six normals used. The two non-absorbers were further tested using a test dose of 120 mg. carotene in 20 gm. butter, and were both found to absorb the pigment. Thus it was concluded that the two patients failed to absorb the carotene when administered with the orange juice alone. It is interesting to note that absorption, when it did occur, was comparable in subjects given the dispersion with and without butter.

TABLE XXV

Carotene Tolerance in Subjects After a Loading Dose of Beta-Carotene Dispersed in Orange Juice[a]

Subject	Serum Carotene in mcg/100 ml. Time in Hours					Max. increase in mcg. percent
	0	4	8	12	24	
A.L.[b]	185	212	218	220	270	85
J.M.[b]	113	132	143	142	172	59
H.W.[b]	87	116	122	115	118	31
A.J.[b]	43	64	57	59	71	28
J.P.[b]	200	220	197	191	228	28
G.G.[b]	117	149	127	142	142	32
P.A.[b]	100	110	113	112	131	31
V.F.[b]	97	129	99	108	129	32
Z.N.[b]	53	65	64	64	76	23
A.M.[c]	52	53	50	52	51	Flat
G.A.[c]	27	31	34	32	37	Flat

[a] Dispersion of 120 mg. of 10% Beta-Carotene Beadlets in orange juice with 20 gm. butter on a slice of bread. Beadlets were supplied courtesy of Hoffmann-La Roche Ltd., Montreal, P.Q.

[b] Known normals.

[c] Known malabsorbers.

TABLE XXVI

Carotene Tolerance in Normal Subjects After a Loading Dose of Beta-Carotene Dispersed in Orange Juice[a]

Subject	Serum carotene in mcg/100 ml. Time in Hours					Max. increase in mcg. percent
	0	4	8	12	24	
L.B.	159	211	207	189	194	52
O.G.	99	112	113	129	131	32
M.G.	106	111	107	99	96	flat
M.K.[b]	88	106	-	-	-	18
L.M.	54	119	104	94	75	65
C.C.	46	43	44	42	49	flat

[a] Dispersion of 120 mg. of 10% Beta-Carotene Beadlets in orange juice without butter. Beadlets were supplied courtesy of Hoffmann-La Roche Ltd., Montreal, P.Q.

[b] Patient discharged.

DISCUSSION

Generally, some variation in stability of crystalline carotene samples was noted throughout the experimental work, giving rise to different oxidation curves. These variations occurred primarily among samples obtained from different sources. In some cases, 50 per cent destruction of carotene occurred after four weeks, while other samples showed only 20 per cent loss in the same period of time under the same conditions. The oxidation curves varied, some showing a plot typical of an autocatalytic process, illustrating the initial induction period, the rapid rate of oxidation, and the gradual termination. Other oxidation curves did not show the initial induction period. Different decomposition rates were also found with samples exhibiting the same initial potencies. This difference, however, was not as pronounced and could be attributed to factors such as temperature variation, type of container used in the study, and the length of time the material was exposed to air during preparation of the samples. Therefore, to minimize variation, the same source of carotene was used for each study. Further precautions, such as storage of the stock material at low temperatures, were observed. As a result, two different series of results were not compared directly, but direct comparisons were made within the same series.

With control over experimental conditions, factors affecting stability of pure carotene were studied. Temperature and oxygen were found to be the major factors to consider in prolonging the shelf-life of carotene. In two weeks, complete destruction of carotene occurred at 50^{o}C, while the refrigerated sample showed only 7.5 per cent loss in potency. Storage of

the pigment at -10°C. showed only a 6.3 per cent loss after eight months, indicating the desirability of this method of storage.

In preliminary studies, carotene was shown to be relatively stable in a nitrogen atmosphere. The stability of the oxidizable compound was also improved by storage in stoppered vials which prevented the diffusion of oxygen into these containers. The total period of stability of the carotene in this stagnant atmosphere, however, could not be estimated. With reference to the tablet study, it was seen that the tablets remained stable for six months even at 50°C. when stored in stoppered containers. Thus it appears that carotene is stable even at relatively high temperatures provided the available oxygen is kept at a minimum.

The effect of diluents and light was found to be negligible. As no adverse effects were observed with the various powdered diluents tested, including lactose, it was concluded that instability of carotene, when stored in a combination with lactose, was not due to the presence of this material. However, storage of carotene in dark capsules did not prolong stability, an observation which was unexpected. The capsules contained dyes which absorbed radiation in the region 400 to 500 mμ. Since the light absorption of carotene occurs in the same region, it would seem that the light rays absorbed by the dyes would result in decreased rate of oxidation. Presumably then, if light does have an effect, it is not sufficient to affect the rate of destruction. Oxidation proceeded at such a rapid rate that the effect of light may have been obscured.

Although some workers have suggested that, in general, carotene should be stored in the dark to increase its stability, the literature revealed no data dealing with the comparative stabilities of the solid pigment in the light and in the dark. On the other hand, it has been shown that solutions of carotene are more stable in the dark than in the light (74).

As indicated previously, carotene in dilute solution has been protected by antioxidants in small concentrations. In this study, antioxidants were found to be ineffective in solution in the quantities employed. The ineffectiveness of the antioxidants in this study may have been due to the large amount of carotene involved, or to the physical state. In the antioxidant study in the dry state, since there was no common medium or substrate to act in, there was no uniform contact between the two solids, and an intimate combination of the two materials was not effected. Carotene has been stabilized in forage by antioxidants. However, the environment involved in the protection of the natural pigment is not comparable to that of the crystalline carotene. When used to protect carotene in the crystalline state, only tocopherol in high concentration was found to exhibit a significant degree of protection. Stability was found to increase with increasing concentrations of the tocopherol. Preliminary testing of the effect of alpha-tocopherol on stability of crystalline carotene at room temperature, indicated a trend as described above. After four weeks, the carotene had lost 50 per cent of its potency when stored without antioxidant, while only 15.8 per cent and 29.3 per cent of the active material had been lost when stored in the presence

of 10 per cent and 5 per cent alpha-tocopherol, respectively. Although stability was apparently increased by the tocopherol, this increase was not considered sufficient to be of practical value for long periods of storage. Also, the practicality of employing such high concentrations of antioxidant was questioned.

Recrystallization as a means of increasing stability by providing a purer product was found to be of no practical value here. The rapid rate of decomposition of the recrystallized product was attributed to the fact that upon recrystallization the carotene took on a finer crystal structure, thus exposing a larger surface area to the atmosphere than the unrecrystallized powder. It should be noted that when recrystallizations were performed, no attempt was made to protect the material from atmospheric oxygen. Recrystallization under nitrogen may have produced a more stable product.

In considering various dosage forms, the practicality of a stable tablet was immediately apparent. It was expected that compression of the powder would reduce the surface area exposed to the air to a degree sufficient to slow the oxidation process. This theory was not borne out, however, as the tablets and crystalline material, when exposed to the air, suffered the same degree of oxidative deterioration.

Coated as well as uncoated tablets proved to be very unstable under these conditions. Tablet coating has long been used as a means of protecting tablets from atmospheric conditions. Film coatings of PEG 6000, and a combination of PVP and carnauba wax failed to protect the active material in the

tablet. Since carotene is so subject to oxidation, a very effective coating material would have to be employed to prevent diffusion of the atmospheric oxygen. The film coats used in this study were ineffective and any protection afforded was insufficient to noticably affect the rate of destruction. Film coatings have been known to allow gaseous penetration and obviously permeation by atmospheric oxygen still occurred. A possible solution would be to coat the tablet with a further sugar coating which would provide a more impervious coat. However, this is a lengthy procedure in which the carotene is subjected to heat and air, thereby allowing a possible initiation of the oxidative process, as seen in the first batch of tablets prepared here. Spraying of the granules before compression is also suggested. Strip packaging may also prove beneficial provided a hermetic seal is produced. These methods should provide significant protection, as it was seen in studies here that the tablets remained stable in air-tight containers for a significant period of time. Since tablets were unsuccessful in clinical studies, further methods of stabilizing this dosage form were not investigated.

Since a satisfactory dosage form in the solid state could not be obtained, liquid preparations were considered. For ease of administration, a standard dose of five ml. (one teaspoonful) was employed. Solubilization of carotene in oil with Spans and Tweens was found not to be possible because of the high concentration of solid material in the required volume of oil. Klaui succeeded in solubilizing carotene in oil (91), however his

success was based on extremely low concentrations of the pigment.

Stability studies of carotene in vegetable oils showed olive and cottonseed oils to be comparable in their protection of the pigment. Corn oil was considered less acceptable. The superiority of the cottonseed over the corn oil was attributed to the presence of natural antioxidants in the former (76). The effectiveness of the olive oil was attributed to its more saturated nature, as it has a lower iodine number than either of the other oils employed. The carotene in the various oils showed better stability than crystalline carotene, presumably because the oil acted as an oxygen barrier.

As indicated previously, an emulsion-type preparation was not physically acceptable. Reduction of particle size by methods such as micropulverization under nitrogen is suggested as a means of obtaining a uniform dispersion. Since carotene is slowly soluble in oil, the emulsion is not physically stable and creaming of the emulsion occurs. Incorporation of carotene into a commercially available fat emulsion did not produce an acceptable preparation as creaming occurred and the coarser particles settled and consequently dispersion was not uniform.

An emulsion was recommended over a suspension initially because of the taste factor. Since a satisfactory emulsion could not be prepared in this laboratory, suspensions were investigated. Stability studies on suspensions showed very little or no decrease in concentration in the time studied. Studies were not continued for a period of sufficient duration to determine the length of stability of the preparations and

the subsequent rate of oxidation at the concentration used. Geminder et al. studied the rate of oxidation of 24 per cent carotene in cottonseed oil at 37.5°C and showed the effectiveness of prolonging the stability of oil preparations of carotene by replacing the headspace of the container with an inert gas (65). After one and one half months, the carotene had lost approximately 5 per cent of its color. In our results, after six weeks, the samples exposed to the air had lost only 6 per cent potency. Since these samples were much more stable than the control when exposed to air, they offered an improvement over the presently used crystalline dose. Preparation of a bulk oil suspension which would allow accurate aliquots to be withdrawn at the required time would provide a more stable dose than the system presently employed.

The availability of water-dispersible beadlets as a stable dosage form prompted a clinical and stability study based on this material. The product is a dispersion of pure beta-carotene and vegetable oil, in a matrix of gelatin and carbohydrate in the form of dark orange-red, free-flowing, spherical beadlets having an irregular surface. The manufacturer claimed excellent stability for the product, attributing this stability to the gelatin-sugar matrix surrounding each beadlet (86). At 45°C., the beadlets exposed to air had retained 100 per cent potency for six weeks. Results in this laboratory, at the same temperature, indicated no loss of the carotene after three weeks. Further studies were discontinued due to time limitations. Thus further confirmation of the stability claims for this product was not possible.

The effect of several selected dosage forms on serum carotene levels was investigated clinically after oral administration. Studies were made in an effort to determine the absorption of the preparation in normal subjects rather than to diagnose patients for the malabsorption syndromes. Most results obtained were inconclusive as non-uniformity prevailed. Generally, it appeared that the distribution of the carotene particles within the vehicle and the presence of butter were the major factors governing efficient absorption of the carotenoid. This was evident from the flat curves obtained when beta-carotene was administered without butter or without being intimately dispersed in the medium. Administration of carotene in solid milk chocolate, hydrogenated vegetable oil, and cocoa butter did not promote absorption. However, administration of the drug with 2 gm. butter (one-tenth of the usual butter load) gave a significant rise in the serum, indicating the value of butter in promoting absorption. It was interesting to note that the powdered material, swallowed in a capsule along with a test meal of bread and butter, was not absorbed by the normal patient. From these observations, it would seem that an intimate dispersion with butter was required.

The importance of a lipid test regimen was especially evident in the tablet study. Butter was seen to have some beneficial effect on the absorption of the carotene given in tablet form. Without butter, no absorption occurred, while the addition of butter enhanced the uptake of the pigment in five of nine subjects. Although the presence of the fat obviously had a significant effect, it was not sufficient to

provide reliable results. The disagreement in results was not readily explainable but oxidation of the carotene while passing through the intestinal tract could have been responsible in part at least. Different absorption patterns among individuals may provide an explanation, but this difference should not be so pronounced as to give such variation in elevation of serum levels as was obtained.

When carotene, in beadlet form, was administered as a dispersion in orange juice, with 20 gm. butter to promote absorption, absorption occurred readily. Comparable serum increases were observed when the dose was given without butter in all patients but two. These results were difficult to explain as it would be expected that lower increments would occur without butter. Also it was difficult to explain the effectiveness of the dispersion as a means of obtaining carotene absorption in all cases but two. It was of interest to note that Cornwell et al. (41), reported flat curves when carotene was given as an aqueous dispersion to two patients. On the other hand, when administered with margarine a very significant increase was observed.

Therefore from studies performed, it seems that no definite conclusions regarding the value of the butter as an aid to absorption of the dispersion of carotene can be made until more studies are performed. It appears, however, that if the test is to produce reliable results, butter must be present.

Although the importance of particle size was not determined on a quantitative or statistical level, it is suggested that further work be done in this area. From preliminary observations it appeared that particle size may have a significant influence on absorption of carotene.

From general observations, it would appear that the Carotene Tolerance Test is very reliable provided it is performed properly. Various factors affecting carotene absorption must be considered before the test is applied. A patient diagnosed by this method must not be suffering from any of the following conditions: hypothyroidism, liver disease, diabetis, or hypercholesterolemia. The patient should not be on steroid therapy. Such patients were avoided in this study, as any of these conditions would make the test unreliable.

In patients studied, it was found that blood samples must be collected at the times indicated, as the increase was seen to vary with individuals. The majority of patients, however, attained the significant rise in 24 hours. In thirty-four normal patients, fasting serum levels ranged from 43 to 200 mcg. per cent, with a mean value of 99 mcg. per cent. Due to different absorption patterns, the degree of absorption was seen to differ quite widely with individual subjects, some exhibiting maximum increases of 85 mcg. per cent, while other normals had a rise of only 18 mcg. per cent. Degree of absorption was comparable in all series, when absorption occurred, regardless of mode of administra-

tion of the drug. Wide variation was evident within each series of normals tested.

Although the clinical results obtained were useful, it must be emphasized that these studies can only be regarded as preliminary work, since numbers involved were relatively small in all series. For that reason, data obtained during the *in vivo* phase of this work are taken as indicative of trends only and firm conclusions cannot be drawn until further studies are made.

SUMMARY AND CONCLUSIONS

1. Various factors affecting stability of crystalline beta-carotene were investigated. It was concluded that temperature and oxygen were the main factors to consider when storing the pigment. Storage at low temperatures or in a limited oxygen atmosphere increased the shelf-life of the compound considerably. Effects of light and diluent materials were found to be negligible. Recrystallization of carotene accelerated rather than decelerated the oxidative process.

2. Although protection of the material by physical methods such as storage at low temperature, or in a limited oxygen atmosphere, was successful, chemical protection by antioxidants was not achieved. This was attributed to the physical state of the system and to the large quantity of the oxidizable compound involved. Alpha-tocopherol in unusually high concentrations was found to exhibit a positive effect on carotene stability.

3. Storage of carotene in an oil vehicle gave a suitable product of improved stability. However, this preparation was considered to be clinically unsatisfactory in that not all of the normal patients tested yielded a significant rise in serum carotene levels.

4. Dosage forms of carotene investigated included tablets, suspensions, and emulsions. Tablets remained stable only when stored in air-tight containers. Clinical studies on these dosage forms gave results that

were less than satisfactory in that at least some of the normal patients tested failed to exhibit a significant elevation in serum carotene.

5. A commercial product, in the form of carotene beadlets, appeared to be relatively stable. When used as a diagnostic drug in the study of malabsorption, it was found to give good rises in normal subjects and flat curves in malabsorbers. Patient acceptibility of the product (administered as a dispersion in orange juice) was very good. Thus, a product of this type would appear to offer promise as a means of solving the stability problem presently encountered in the use of carotene as a diagnostic agent. Further study of the material, particularly the clinical aspects, would appear to be indicated.

6. Since reduction of particle size has been recommended as a means of increasing degree of absorption of carotene, it is suggested that formulation of a tablet containing microcrystalline carotene be investigated. Such a tablet would have to be prepared under nitrogen by the manufacturer, and stored individually in sealed containers to insure stability.

BIBLIOGRAPHY

1. Cayer, D., J.M. Ruffin and W.A. Perlzweig, Amer. J. Med. Sci., 210, 200-207, 1945.

2. Sebrell, W.H. and R.S. Harris, "The Vitamins", Academic Press Inc., New York, Vol. 1, p.4, 1954.

3. Goodwin, T.W.,"Chemistry and Biochemistry of Plant Pigments", Academic Press Inc., New York, p. 490, 1965.

4. Wackenroder, Geigers Magazin Pharm., 33, 141, 1831. From Karrer, P. and E. Jucker, "Carotenoids", Elsevier Pub. Co. Inc., Amsterdam, p. 126, 1950.

5. Burger, A., "Medicinal Chemistry", Interscience Pub. Inc., New York, Vol. 1, pp. 513-516, 1951.

6. Willstatter, R. and W. Mieg, Ann., 355, 1, 1907. From Karrer, P. and E. Jucker, "Carotenoids", Elsevier Pub. Co. Inc., Amsterdam, p. 126, 1950.

7. Karrer, P., Helv. Chim. Acta, 14, 1033-1046, 1931. From Karrer, P. and E. Jucker, "Carotenoids", Elsevier Pub. Co. Inc., Amsterdam, p. 126, 1950.

8. Kuhn, R. and H. Brockmann, Ann. 516, 95, 1935. From Karrer, P. and E. Jucker, "Carotenoids", Elsevier Pub. Co. Inc., Amsterdam, p. 126, 1950.

9. Karrer, P. and C.H. Eugster, Helv. Chim. Acta., 33, 1172, 1950.

10. Karrer, P. and E. Jucker, "Carotenoids", Elsevier Pub. Co. Inc. Amsterdam, p. 135, 1950.

11. Lundberg, W.O., "Autoxidation and Antioxidants", Intercience Pub. Inc., New York, Vol. 1, p.2, 1961.

12. Karrer, P. and E. Jucker, op. cit., pp. 6-7.

13. Carr, F.H. and E.A. Price, Biochem. J., 20, 497, 1926.

14. Deuel, H.J., "Lipids", Interscience Pub. Inc., New York, Vol. 1, pp. 602-606, 1951.

15. Sebrell, W.H. and R.S. Harris, op. cit., p. 15.

16. Zechmeister, R. and A. Polgar, J. Amer. Chem. Soc., 65, 1522-1528, 1943.

17. Luckmann, F.H., D. Melnick and H.W. Vahlteich, J. Amer. Oil Chem. Soc., 29, 321-330, 1952.

18. Ralli, E.P., H. Brandaleone and T. Mandelbaum, J. Lab. Clin. Med., 20, 1266-1275, 1935.

19. Murrill, W.A., P.B. Horton, E. Leiberman and L.W. Newburgh, J. Clin. Investig., 20, 395, 1941.

20. Kraybill, H.R. and C.L. Shrewsbury, J. Nutr., 11, 103-110, 1936.

21. Roels, O.A., M. Trout and R. Dujacquier, J. Nutr., 65, 115-127, 1958.

22. Ehrlich, S.T., B.R. Farthing and D.S. Moschette, J. Nutr., 84, 389-394, 1964.

23. Curtis, A.C. and E.M. Kline, Arch. Int. Med., 63, 53-62, 1939.

24. Thompson, S.Y. "Carotine und Carotenoide" (K. Lang, editor), Wissenschaftliche Veröffentlichungen der Deutschen Gesell-für Ernährung, 9, 263-277, 1961.

25. Shaw, R.J. and H.J. Deuel, J. Nutr., 27, 395-401, 1944.

26. Kon, S.K., W.A. McGillivray and S.Y. Thompson, Brit. J. Nutr., 9, 244-267, 1955.

27. Adlersberg, D., S. Kann, A.P. Maurer, K. Newerly, W. Winternitz and H. Sobotka, Amer. J. Dig. Dis., 16, 333-336, 1949.

28. Harris, P.L., M.W. Kaley and K.C.D. Hickman, J. Biol. Chem. 152, 313-320, 1944.

29. Moore, T., Biochem. J., 23, 803-810, 1929.

30. Glover, J., T.W. Goodwin and R.A. Morton, Biochem. J., 43, 512-517, 1948.

31. Olson, J.A., J. Biol. Chem., 236, 349-356, 1961.

32. Thompson, S.Y., J. Ganguly and S.K. Kon, Brit. J. Nutr., 3, 50-76, 1949.

33. Bieri, J.G. and C.J. Pollard, Brit. J. Nutr., 8, 32-43, 1954.

34. McGillivray, W.A., S.Y. Thompson and N.A. Worker, Brit. J. Nutr., 10, 126-134, 1956.

35. Worker, N.A., Brit. J. Nutr., 13, 400-417, 1959.

36. Olson, J.A., J. Lipid Res., 5, 281-295, 1964.

37. Crain, F.D., F.J. Lotspeich and R.F. Krause, Proc. Soc. Exp. Biol. Med., 119, 606-608, 1965.

38. Drummond, J.C., M.E. Bell and E.T. Palmer, Brit. Med. J., 1, 1208-1210, 1935.

39. Thompson, S.Y., R. Braude, M.E. Coates, A.T. Cowie, J. Ganguly and S.K. Kon, Brit. J. Nutr., 4, 398-420, 1950.

40. Krinsky, N.I., D.G. Cornwell and J.L. Oncley, Arch. Biochem. Biophys., 73, 233-246, 1958.

41. Cornwell, D.G., F.A. Kruger and H.B. Robinson, J. Lipid Res. 3, 65-70, 1962.

42. Deuel, H.J., "Lipids", Interscience Pub. Inc., New York, Vol. 1, p. 254, 1951.

43. Urbach, C., K. Hickman and P.L. Harris, Exp. Med. Surg., 10, 7-18, 1952.

44. Bockus, H.L.,"Gastroenterology", W.B. Saunders Co., Vol. 2, p. 425, 1964.

45. Texter, E.C., J.A. Cooper, M. Vidlini and J. M. Finlay, Med. Clin. N. Amer., 48, 117, 1964.

46. Ruffin, J., Amer. J. Dig. Dis., 6, 1054, 1961.

47. Parker, J.G. and G. Ross, Amer. J. Dig. Dis., 5, 790, 1960.

48. Wenger, J., J.B. Kirsner and W.L. Palmer, Amer. J. Med., 22, 373-379, 1957.

49. Zbinden, G. and A. Studer, Z.Lebensm.-Untersuch u-Forsch, 108, 113-134, 1958. Through Chem. Abst., 53, 594g, 1959.

50. Campbell, D.J., Private Communication, University of Alberta Hospital, Edmonton.

51. Feldman, E.B. and D. Adlersberg, Amer. J. Med. Sci., 238, 733-738, 1959.

52. Ruffin, J., Amer. J. Dig. Dis., 6, 1050-1055, 1961.

53. Budowski, P. and A. Bondi, Arch. Biochem. Biophys., 89, 66-73, 1960.

54. Ostendorf, J.P., J. Soc. Cos. Chem., 16, 203-220, 1965.

55. Duquenois, P. and M. Meylaender, Ann. Fals. Exp. Chim., 56, 371-375, 1963.

56. Schou, S.A. and J. Morch, "Report on Stability of Pharmaceutical Preparations", p. 20, 1959. Reprinted from Archiv for Pharmaci og Chimi (1959).

57. Scott, G., "Atmospheric Oxidation and Antioxidants", Elsevier Pub. Co., Amsterdam, p. 116, 1965.

58. Ibid., p. 363.

59. Food and Drugs Act and Regulations, as amended to Dec. 8, 1965. Dept. Natl. Health and Welfare, Canada.

60. High, E.G., Arch. Biochem. Biophys., 60, 456-462, 1956.

61. Wall, M.E. and E.G. Kelly, Ind. Eng. Chem., 38, 215-218, 1946.

62. MacDonald, F.G., J. Biol. Chem., 103, 455-460, 1933.

63. Wall, M.E. and E.G. Kelly, Ind. Eng. Chem., 43, 1146-1150, 1951.

64. Schnetzer, L., Mitt. Lebensm. Hyg., 46, 90-96, 1955. Through Chem. Abst., 49, 9189f, 1955.

65. Geminder, J.J. and E.E. MacDonough, J. Amer. Oil Chem. Soc., 34, 314-318, 1957.

66. Mills, R.C., and E.B. Hart, J. Dairy Sci., 28, 1-12, 1945.

67. Kitagawa, S. and M. Matsumuro, Ann. Rept. Natl. Inst. Nut. (Tokyo), 78, 1960. From "Stability and Stability Testing of Pharmaceuticals", Pharm. Manuf. Assoc. Pub., Washington, D.C., 1964.

68. Thompson, C.R., Ind. Eng. Chem., 42, 922-925, 1950.

69. Bickoff, E. and K.T. Williams, Ind. Eng. Chem., 36, 320-323, 1944.

70. Bickoff, E.M., J. Amer. Oil Chem. Soc., 28, 65-68, 1951.

71. Bickoff, E. and K.T. Williams, J. Amer. Oil Chem. Soc., 23, 65-68, 1946.

72. Bickoff, E., A.L. Livingston and C.R. Thompson, J. Amer. Oil Chem. Soc., 32, 64-68, 1955.

73. Morgal, P.W., L.W. Byers and E.J. Miller, Ind. Eng. Chem., 35, 794-797, 1943.

74. Baumann, C.A. and H. Steenboch, J. Biol. Chem., 101, 561-572, 1933.

75. Bickoff, E.M., G.M. Coppinger, A.L. Livingston and T.W. Campbell, J. Amer. Oil Chem. Soc., 29, 51-52, 1952.

76. Bickoff, E., K.T. Williams and M. Sparks, J. Amer. Oil Chem. Soc. 22, 128-131, 1945.

77. Bickoff, E., Science, 97, 96, 1943.

78. Williams, K.T., E. Bickoff and B. Lowrimore, J. Amer. Oil Chem. Soc., 21, 161-164, 1944.

79. Siedler, A.J., E. Enzer and B.S. Schweigert, J. Agr. Food Chem., 4, 1023-1029, 1956.

80. Denton, A.E. and E.R. Bielanski, J. Agr. Food Chem., 6, 853-855, 1958.

81. Ogden, R.L., J. Agr. Food Chem., 4, 428-431, 1956.

82. Bickoff, E. and K.T. Williams, Ind. Eng. Chem. Anal. Ed., 15, 266-268, 1943.

83. Fraps, G.S. and A.R. Kemmerer, Chem. Eng. News, 19, 846-847, 1941.

84. Bauernfeind, J.C., E.G. Smith and R.H. Bunnell, Food Technol., 12, 527, 1958.

85. Isler, O., A. Ofner and G.F. Siemers, Food Technol., 12, 520, 1958.

86. Bunnel, R.H., W. Driscoll and J.C. Bauernfeind, Food Technol., 12, 536-541, 1958.

87. Horwitz, W., Official Methods of Analysis of the Assoc. Off. Agr. Chem., Assoc. Off. Agr. Chem. Pub., Washington, D.C., 10th Ed., p. 757, 1956.

88. Lachman, L., J. Pharm. Sci., 54, 1519, 1965.

89. Gans, E.H. and L. Chavkin, J. Amer. Pharm. Assoc. Sci. Ed., 43, 483-485, 1954.

90. Ahsan, S.S. and S.M. Blaug, Drug Standards, 26, 30, 1958.

91. Klaui, H. "Carotine und Carotenoide" (K. Lang, editor), Wissenschaftliche Veröffentlichungen der Deutschen Gesellschaft für Ernährung, 9, 390-398, 1961.

APPENDIX

TABLE I

Effect of Temperature on Stability of Crystalline Beta-Carotene[a]

Time in Days	5°C		25°C		37.5°C		50°C	
	O.D.[b]	$E^{1\%}_{1cm.}$[c]	O.D.[b]	$E^{1\%}_{1cm.}$[c]	O.D.[b]	$E^{1\%}_{1cm.}$[c]	O.D.[b]	$E^{1\%}_{1cm.}$[c]
1	.480	2400	.475	2375	.470	2350	.469	2345
	.480	2400	.475	2375	.475	2375	.470	2350
2	.480	2400	.470	2350	.448	2240	.400	2000
	.480	2400	.470	2350	.450	2250	.400	2000
3	.480	2400	.460	2300	.420	2100	.340	1700
	.480	2400	.458	2290	.430	2150	.340	1700
4	.473	2365	.450	2250	.394	1970	.270	1350
	.474	2370	.450	2250	.395	1975	.270	1350
6	.475	2375	.440	2200	.340	1700	.190	950
	.470	2350	.437	2185	.340	1700	.193	965
8	.470	2350	.428	2140	.293	1465	.105	525
	.470	2350	.430	2150	.289	1445	.105	525
10	.470	2350	.410	2050	.232	1160	.670	350
	.470	2350	.408	2040	.229	1145	.080	400
14	.453	2245	.360	1800	.160	800	-	-
	.458	2290	.363	1815	.165	825		

[a] Samples (100 mg.) were stored in open glass vials

[b] Optical density of carotene in chloroform was determined at 466 mμ. Initial O.D. was .490

[c] Initial $E^{1\%}_{1cm.}$ was 2450

TABLE II

Effect of Atmospheric Oxygen on Stability of Crystalline Beta-Carotene[a]

Time in Weeks	Carotene Exposed to the Atmosphere		Carotene Stored in Closed Vials	
	O.D.[b]	$E^{1\%}_{1cm.}$[c]	O.D.[b]	$E^{1\%}_{1cm.}$[c]
1	.470	2350	.472	2360
	.463	2315	.472	2360
	.463	2315	.472	2360
2	.440	2200	.445	2225
	.430	2150	.440	2200
	.430	2150	.440	2200
3	.400	2000	.410	2050
	.400	2000	.410	2050
	.394	1970	.410	2050
4	.340	1700	.400	2000
	.338	1690	.400	2000
	.348	1740	.400	2000
5	.234	1170	.395	1975
	.236	1180	.392	1960
	.245	1225	.393	1965
6	.182	910	.381	1905
	.191	955	.382	1910
	.192	960	.383	1915

[a] Samples (100 mg.) were stored at room temperature in two dram glass vials.

[b] Optical density of carotene in chlorofrom was determined at 466 mμ. Initial O.D. was .490.

[c] Initial $E^{1\%}_{1cm.}$ was 2450.

TABLE III

Effect of Diluents on Stability of Crystalline Beta-Carotene[a]

Time in Weeks	Control		α-Lactose		Kaolin		Calcium Carbonate		Calcium Phosphate	
	O.D.[b]	$E^{1\%}_{1cm.}$[c]	O.D.[b]	$E^{1\%}_{1cm.}$[c]	O.D.[b]	$E^{1\%}_{1cm.}$[c]	O.D.[b]	$E^{1\%}_{1cm.}$	O.D.[b]	$E^{1\%}_{1cm.}$[c]
1	.810	2025	.814	2036	.808	2020	.820	2050	.823	2057
	.810	2025	.812	2030	.810	2025	.820	2050	.820	2050
2	.608	1520	.610	1525	.630	1575	.604	1510	.628	1570
	.610	1525	.607	1518	.620	1550	.600	1500	.628	1570
3	.510	1275	.495	1238	.498	1245	.532	1330	.472	1180
	.500	1250	.495	1238	.488	1220	.523	1307	.471	1177
4	.330	825	.346	865	.331	828	.364	910	.330	825
	.321	803	.347	868	.327	817	.366	915	.333	829

[a] Samples were stored in clear gelatin capsules at room temperature.

[b] Optical density of carotene in chloroform was determined at 466 mμ. Initial O.D. was .850.

[c] Initial $E^{1\%}_{1cm.}$ was 2125.

TABLE IV

Effect of Light on Stability of Crystalline Beta-Carotene[a]

Time in Weeks	Capsule Color					
	Clear		Red		Brown	
	O.D.[b]	$E^{1\%}_{1cm.}$[c]	O.D.[b]	$E^{1\%}_{1cm.}$[c]	O.D.[b]	$E^{1\%}_{1cm.}$[c]
2	.420	2100	.428	2140	.470	2350
	.420	2100	.428	2140	.455	2275
	.420	2100	.431	2155	.458	2290
3	.358	1925	.390	1950	.390	1950
	.388	1940	.390	1950	.390	1950
	.390	1950	.385	1925	.388	1940
4	.307	1535	.305	1525	.307	1535
	.310	1550	.304	1520	.307	1535
	.310	1550	.310	1550	.307	1535
5	.240	1200	.240	1200	.250	1250
	.243	1215	.240	1200	.238	1190
	.241	1205	.245	1225	.245	1225
6	.175	875	.180	900	.203	1015
	.174	870	.181	905	.191	955
	.184	920	.181	905	.189	945

[a] Samples (120 mg.) were stored at room temperature.

[b] Optical density of carotene in chloroform was determined at 466 mμ. Initial O.D. was .490.

[c] Initial $E^{1\%}_{1cm.}$ was 2450.

TABLE V

Effect of High Concentration of Alpha-Tocopherol on Stability of Crystalline Beta-Carotene[a]

Time in Days	Control		5% Tocopherol		10% Tocopherol		25% Tocopherol	
	O.D.[b]	$E^{1\%}_{1cm.}$[c]	O.D.[b]	$E^{1\%}_{1cm.}$[c]	O.D.[b]	$E^{1\%}_{1cm.}$[c]	O.D.[b]	$E^{1\%}_{1cm.}$[c]
2	.420	2100	.425	2125	.445	2225	.445	2225
	.421	2105	.430	2150	.446	2230	.447	2235
3	.355	1775	.408	2040	.415	2075	.425	2125
	.362	1810	.370	1850	.415	2075	.415	2075
4	.270	1350	.310	1550	.360	1800	.390	1850
	.270	1350	.310	1550	.360	1800	.390	1850
6	.180	900	.210	1050	.241	1205	.290	1450
	.180	900	.210	1050	.240	1200	.290	1450

[a] Samples (100 mg.) were stored in open vials.

[b] Optical density of carotene in chloroform was determined at 466 mμ. Initial O.D. was .490.

[c] Initial $E^{1\%}_{1cm.}$ was 2450.

TABLE VI

Comparative Rates of Oxidation of Recrystallized[a] and Unrecrystallized Beta-Carotene[b]

	37.5°C					Ambient Temperature			
Time in Weeks	Control O.D.[c]	Control $E^{1\%\ d}_{1cm.}$	Time in Days	Recrystallized O.D.[c]	Recrystallized $E^{1\%\ d}_{1cm.}$	Time in Days	Control O.D.[c]	Control $E^{1\%\ d}_{1cm.}$	Recrystallized O.D.[c]	Recrystallized $E^{1\%\ d}_{1cm.}$
1	.772 .773	1930 1933	3	.720 .720	1800 1800	1	.916 .898	2275 2245	.680 .681	1700 1702
2	.510 .520	1275 1300	6	.408 .406	1020 1015	2	.870 .871	2175 2178	.382 .382	955 955
4	.180 .180	450 450	9	.212 .208	530 520	4	.724 .728	1810 1820	.005 .004	12.5 10.0
5	.118 .114	295 285	12	.110 .110	275 275	6	.498 .510	1245 1275	-	-
6	.020 .020	50 50	-	-	-	8	.237 .237	593 593	-	-

[a] Carotene was recrystallized from a chloroform-methanol system.

[b] Samples (100 mg.) were stored in open vials.

[c] Optical density of carotene in chloroform was determined at 466 mμ. Initial O.D. was .960.

[d] Initial $E^{1\%}_{1cm.}$ was 2400.

TABLE VII

Comparative Rates of Oxidation of Recrystallized[a] and Unrecrystallized Beta-Carotene at -10°C.[b]

Time in Weeks	Recrystallized		Unrecrystallized (Control)	
	O.D.[c]	$E^{1\%\ d}_{1cm.}$	O.D.[c]	$E^{1\%\ d}_{1cm.}$
1	.920	2300	.945	2385
	.924	2310	.956	2390
2	.880	2200	.944	2360
	.880	2200	.945	2363
4	.870	2175	.940	2350
	.850	2125	.950	2375
6	.860	2150	.960	2400
	.860	2150	.940	2350
8	.850	2125	.950	2375
	.844	2110	.950	2375
12	.800	2000	.920	2300
	.800	2000	.910	2275
16	.770	1925	.900	2250
	.750	1875	.900	2250
24	.490	1225	.900	2250
	.491	1227	.904	2260
32	.418	1045	.900	2250
	.417	1043	.900	2250

[a] Carotene was recrystallized from a chloroform-methanol system.

[b] Samples (100 mg.) were stored in open vials.

[c] Optical density of carotene in chloroform was determined at 466 mμ. Initial O.D. was .960.

[d] Initial $E^{1\%}_{1cm.}$ was 2400.

TABLE VIII

Stability of Beta-Carotene in Selected Vegetable Oils at 50°C.[a]

Time in Hours	Vegetable Oil							
	Control		Corn Oil		Cottonseed Oil		Olive Oil	
	O.D.[b]	$E^{1\%}_{1cm.}$[c]	O.D.[b]	$E^{1\%}_{1cm.}$[c]	O.D.[b]	$E^{1\%}_{1cm.}$[c]	O.D.[b]	$E^{1\%}_{1cm.}$[c]
24	.432	2160	.460	2300	.480	2400	.480	2400
	.432	2160	.460	2300	.480	2400	.480	2400
			.460	2300	.474	2370	.480	2400
48	.354	1770	.450	2250	.450	2250	.458	2290
	.354	1770	.450	2250	.450	2250	.453	2265
			.444	2220	.448	2240	.448	2240
72	.240	1200	.380	1900	.438	2190	.405	2025
	.238	1190	.375	1875	.433	2165	.405	2025
			.370	1850	.417	2085	.405	2025
120	.120	600	.240	1200	.327	1635	.318	1590
	.120	600	.240	1200	.320	1600	.312	1560
			.280	1400	.300	1500	.300	1500
168	.073	365	.154	770	.200	1000	.202	1010
	.073	365	.167	835	.203	1015	.202	1010
			.171	855	.210	1050	.196	980

[a] Samples (20 mg. per ml. oil) were stored in open vials.

[b] Optical density of carotene in chloroform was determined at 466 mμ. Initial O.D. was .490.

[c] Initial $E^{1\%}_{1cm.}$ was 2450.

TABLE IX

Stability of Beta-Carotene in Cottonseed Oil at Room Temperature[a]

Time in Weeks	Suspension in Closed Container		Suspension Exposed to Air	
	O.D.[b]	$E^{1\%\ c}_{1cm.}$	O.D.[b]	$E^{1\%\ c}_{1cm.}$
1	.488	2440	.480	2400
	.490	2450	.472	2360
	.489	2445	.490	2450
2	.475	2375	.480	2400
	.488	2440	.480	2400
	.488	2440	.490	2450
3	.485	2425	.465	2325
	.480	2400	.480	2400
	.490	2450	.475	2375
4	.463	2315	.490	2450
	.480	2400	.480	2400
	.485	2425	.463	2315
6	.480	2400	.460	2300
	.468	2340	.462	2310
	.455	2275	.460	2300

[a] Cottonseed oil was carried as a blank. Crystalline carotene in closed containers, and carotene exposed to the air served as controls. Values obtained were previously reported (See Table II).

[b] Optical density of carotene in chloroform was determined at 466 mμ. Initial O.D. was .490.

[c] Initial $E^{1\%}_{1cm.}$ was 2450.